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GOLD IN RHEUMATOID ARTHRITIS: ITS EFFECT ON PLASMA CORTISOL AND ITS DEPOSITION IN SKIN.

By

© Dorothy A. Jeffery, M.Sc.

A THESIS

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled GOLD IN RHEUMATOID ARTHRITIS: ITS EFFECT ON PLASMA CORTISOL AND ITS DEPOSITION IN SKIN submitted by Dorothy A. Jeffery in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Biopharmacy.

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ABSTRACT

The role of the adrenal cortex in the action of gold therapy in rheumatoid arthritis has been investigated by determination of cortisol levels, cortisol binding to plasma proteins and transcortin binding capacity and affinity in patients' plasma. No effect of gold therapy on these parameters could be found in patients newly initiated to chrysotherapy before or during gold treatment, in matched patients where one patient was receiving gold while the other was receiving other forms of therapy for rheumatoid arthritis, or in patients at various stages of gold therapy as measured by plasma gold level.

For the study of transcortin binding parameters, a competitive adsorption technique was developed by modification of previous methods. Approaches to resolution of binding parameters from data for two binding components, have been considered and computer programs for resolving this type of data have been written.

Skin, hair, nail and blood gold levels were determined by flameless atomic absorption spectroscopy in patients who had received large total doses of gold. Higher skin gold levels, ranging from 53 to 140 mcg/g were found in specimens

from patients exhibiting chrysiasis, than in patients not showing chrysiasis who had skin gold levels ranging from 7.5 to 38 mcg/g. In the latter non-chrysiasis group, skin gold levels were strongly correlated with gold dose rate, expressed as mg gold per week. Chrysiasis patients had higher skin gold levels than expected based on their gold dose rate. Concentrations of gold in hair and nails were 0.2 to 0.7 and 1 to 4 mcg/g, respectively. Plasma or serum gold levels were 0.6 to 2.9 mcg/g while red blood cell gold was 0.15 to 0.9 mcg/g.

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INTRODUCTION

Gold has a long history of use in medicine. Ancient man from several cultures thought gold had the ability to prolong life. Pliny recorded early medical uses of gold as a cure for lichens of the face, hemorrhoids, warts, putrid sores, ulcers, and as a purgative (Smit, 1968). Through the ages gold continued to be valued as a medicine. In the 1918 United States Dispensatory gold was listed as being useful in treatment of chronic nervous disorders, epilepsy, alcoholism, hysteria, chronic nutritional disorders, anemia, dysmenorrhea, ovarian irritation, chronic uterine inflammation, syphilis, lymphatic swelling, lupus, diabetes mellitus, tuberculosis and chronic rheumatism. There was little rational basis for any of these uses. Today gold is of lesser importance in medicine, its chief use being in the treatment of rheumatoid arthritis, although it is also used as gold leaf in the treatment of decubitus ulcers, and as ^{198}Au in nuclear medicine (Smit, 1968).

The value of gold therapy in rheumatoid arthritis has been controversial but a multicentre study by the Empire Rheumatism Council (1961a), recently confirmed by a double blind study in the United States (Cooperating Clinics Committee, 1973) has established that gold is effective in controlling rheumatoid arthritis. The mechanism by which gold produces this beneficial effect is still unknown. Many preparations of gold have been developed with the aim of optimizing the therapeutic ratio; however it appears that it is the content of gold which is responsible for both the therapeutic and the toxic effect of the preparations (Freyberg, 1966). Today gold sodium thiomalate and aurothioglucose are used almost exclusively in Canada and the United States, while gold sodium thiosulfate is used in some parts of Europe. All these compounds must be given parenterally. Recently Smith Kline and French have prepared chloro(triethylphosphine)gold which is well absorbed orally and is as effective as intramuscular gold sodium thiomalate in suppressing the secondary lesions of adjuvant arthritis in rats (Walz, 1972).

Toxicity to gold therapy is quite high (5-50%) (Meyler, 1968);however with careful monitoring of patients chrysotherapy is quite safe since relatively minor side effects usually precede serious toxicity. Two aspects of gold toxicity form the basis for this thesis. First, toxicity has frequently been associated with a successful response to gold therapy (Bayles, 1956; Copeman, 1937; Freyberg, 1966; Krusius, 1970). This may be meaningful in terms of the mode of action of gold , which may be via a stress reaction and a subsequent action on the adrenocortical system. Secondly, one of the rare side effects of gold is chrysiasis, a pigmentation of the skin (Everett, 1972). The cause of this pigmentation has not been established. It may be the actual deposition of gold in the skin. These two aspects of gold therapy form the basis of the following investigation.

SURVEY OF LITERATURE AND THEORETICAL CONSIDERATIONS

A. POSSIBLE MECHANISMS OF ACTION FOR GOLD

The mechanism of action of gold in rheumatoid arthritis is not known; however many different hypotheses have been suggested. The etiology of rheumatoid arthritis is unknown and this further complicates explanation of the beneficial effects of gold. Theories which have been put forward to account for the etiology of the disease have been reviewed by a number of authors (Weissmann, 1972; Zvaifler, 1965; Zvaifler, 1970; Rodnan, 1973; Ziff, 1973). From these reveiws it appears that the theory that rheumatoid arthritis is an auto-immune disease or an immunological disease initiated by some unidentified infective agent can partially explain the pathology. Some unknown initiating agent which may be an infectious agent (eg mycoplasma or a virus); possibly a denatured immunoglobulin (eg IgG from the joint) acts as a stimulus for the formation of IgG in the joint. This IgG stimulates the production of rheumatoid factor in the joint, and later interacts with it forming immune complexes which fix and activate complement leading to liberation of various active products including chemotactic substances. These attract polymorphonuclear leukocytes (PMN) which ingest the immune complexes and in some way release lysosomal hydrolase enzymes. These enzymes cause inflammatory destruction and proliferative changes in the joints perhaps producing partially denatured proteins which act as antigens stimulating the further synthesis of IgG and perpetuating the cycle of destruction even after the disappearance of the initial cause. Large amounts of fibrin, fibrin split products and fibrinogen are also present in inflamed joints and are ingested by PMN. Gold may act at some point in this cycle or may act from another part of the body and interrupt the cycle thus producing its beneficial effects. With this brief theory of the causation of rheumatoid arthritis (RA) as a framework, previous investigations which have been aimed at elucidation of the mode of action of gold will be discussed.

Gold compounds have a bacteriostatic effect against several bacterial species (Mycobacterium tuberculosis, Staphylococci, Streptococci, Spirochaetes, Mycoplasmas) (Nineham, 1963 Hartung, 1941a; Davidson, 1966; Stewart, 1969; Marmion, 1961). The antibacterial activity of gold is usually reduced in the presence of blood or plasma probably due to protein binding of gold (Nineham, 1963). Treatment of infections in most cases requires near toxic doses (Nineham, 1963). Gold sodium thiomalate can prevent but not cure hemolytic streptococcal arthritis in rats (Rothbard, 1941). Gold compounds can cure mycoplasmal induced arthritis but this cure is not due antibacterial action since gold sodium thiomalate in this study failed to inhibit growth of mycoplasma in cultures nor did it attenuate the arthritis producing potential of mycoplasma grown for several generations in the presence of gold (Sabin, 1940). The necessity of gold in the molecule for anti-arthritic activity was shown by Preston (1942) using this model of arthritis. Gold sodium thiomalate (Au-S-) and gold succinimidoaurate (Au-N<) were able to cure the arthritis, while nongold analogues (sodium thiomalate, disulfide sodium thiomalate) could not.

Gold sodium thiomalate does not affect immune responses in treated animals in certain types of assays. Delayed hypersensitivity reaction to diphtheria toxoid and dinitrochlorobenzene in guinea pigs; delayed skin reaction to tuberculin in arthritic rats; circulating antibodies in rabbits immunized with bovine serum albumin, typhoid-paratyphoid vaccine or Escherichia coli, or in arthritic rats immunized with sheep red blood cells were not affected by gold (Jessop, 1968; Persellin, 1967). Synthesis of antibodies against bovine serum albumin by lymphoid cells isolated from immunized rabbits is inhibited by gold sodium thiomalate provided pre-incubation of cells with gold was done at 37°C, not at 0°C. Cells were equally viable when either pre-incubation temperature was used, so it was claimed that active transport of gold into the cells was required for this effect (Persellin, 1963). Lymphoid cells isolated from gold treated mice immunized with sheep red blood cells showed a marked increase in antibody production whereas if gold treatment preceded immunization antibody production was slightly suppressed. It appears the response of lymphoid cells to gold is phasic, initial stimulation followed by suppression (Scheiffarth, 1971). This phasic effect likely explains contradictory reports (Keszthelyi, 1956; Norn, 1968a,b). Gerber (1972d) has studied the immunosuppressive effect of gold in rats using an immunologically mediated disease (experimental allergic encephalomyelitis, EAE). Gold treatment could delay or ameloriate EAE apparently by acting at some stage after the initial sensitization with the encephalitogen. In humans rheumatoid factor becomes negative in approximately 40% of RA patients treated with gold (Klinefelter, 1973). This may be related to the immunologic action of gold or may be an indirect effect. It appears that gold does possess some immunosuppressive activity but this is much less than that of cyclophosphamide or methotrexate.

Immune complex formation in the rheumatoid joint leads to activation of complement and liberation of biologically active substances. Mizushima (1965) has shown that ionized gold (as $AuCl_3.HCl$) has anti-complementary activity at gold concentrations of 10^{-4}M. These workers also showed that aurothioglucose inhibits the activity of a necrotizing factor which may be one of the active substances released during immune complex formation. Another active substance released during the antigen antibody reaction is histamine. *In vitro* release of histamine by isolated rat peritoneal mast cells challenged with specific antigen is inhibited if the animal is pre-treated, during sensitization, with gold sodium thiosulfate (25 mg/kg/d x 3 d). The effect is not one of decreased antibody production since serum from treated rats can passively sensitize untreated rats, nor is it due to decreased concentration of mast cells or decreased histamine content of the cells. Inhibition of histamine release must occur through inhibition of the antigen antibody reaction, perhaps by inhibition of some enzymes involved (Norn, 1965, 1968b). The response of isolated guinea pig jejunum to histamine is inhibited by $2.2x10^{-4}$M gold sodium thiomalate, suggesting an antihistaminic effect of gold (Trethewie, 1970).

Polymorphonuclear leukocytes which are attracted to the rheumatoid joint by chemotactic substances, carry out phagocytosis. Gold sodium thiomalate ($5x10^{-6}$M) has been shown to inhibit phagocytosis of starch granules by PMN (Chang, 1969). Jessop (1973b) has devised a 'skin-window' technique for *in vivo* assessment of phagocytic activity of macrophages and PMN in humans. Using this technique he showed that phagocytic activity was elevated in RA but was lowered to normal by gold therapy.

Phagocytosis of immune complexes by PMN may lead to damage to lysosomes and increased release of hydrolase enzymes which cause destruction of tissue. Rheumatoid synovial membrane has been shown to have elevated lysosomal enzyme activity compared to normal tissue (Goldfischer, 1967). Gold is known to inhibit the activity of many enzymes probably through reaction with sulfhydryl groups in the enzymes (Table 1). The activity of lysosomal enzymes, such as acid phosphatase, beta-glucuronidase, cathepsin, is inhibited by gold concentrations readily attainable during therapy. The reduced tissue destruction, resulting from inhibition of lysosomal enzymes by gold and hydrocortisone, was shown with chick synovial cartilage biosynthetically labelled with ^{35}S. These drugs reduced the release of ^{35}S caused by the destruction of cartilage by lysosomal enzymes from rheumatoid synovia (Hawkins, 1972). Persellin (1966) showed that macrophages incubated at 37°C with gold took up gold, as evidenced by inhibition of enzyme activity. The process of uptake appears active since incubation at 0°C gave no inhibition of enzyme activity. Subcellular fractionation showed most of the acid phosphatase and beta-glucuronidase activity to be in the 15000g pellet (lysosomes and mitochondria) compared to the nuclear fraction and supernatant. The enzyme activity of the 15000g pellet was markedly reduced on incubation with gold; while activity in the supernatant and nuclear fraction was little affected. This was taken as evidence of uptake of gold into the organelles of the 15000g fraction (likely lysosomes). The mitochondrial enzyme malic dehydrogenase was also inhibited, but this was not felt to be evidence of mitochondrial uptake of gold. Rather it was interpreted to be contamination of mitochondria with gold from lysosomes lysed as part of preparation for enzyme assay. Paltemaa (1968b) suggested that gold is accumulated in damaged lysosomes of rheumatoid tissue more readily than in normal lysosomes. Lysosomal damage can be produced experimentally in rats by vitamin A. After this type of treatment to damage the lysosomes, intracellular gold concentration paralleled damage to lysosomes, measured by acid phosphatase activity in isolated liver cells. In contrast to these workers who suggest gold uptake into the lysosome is needed for maximum inhibition of hydrolases, Lorber (1970) found only extracellular acid hydrolases were inhibited by gold.

A decreased destruction of tissue by lysosomal enzymes could also result from stabilization of the lysosomal membrane and thus lesser release, rather than or as well as a direct inhibition of the enzymes. However, biochemical studies indicate gold does not act in this manner (Ennis, 1968; Ignarro, 1971). Other evidence from lymphocyte cultures suggests gold may act to stabilize lysosomal membranes. Lymphocytes, which do not normally divide, when challenged with phytohemagglutinin (PHA) or streptolysin-S (SLS), are transformed into blast cells. It is postulated that blast cell transformation may be triggered by the rupture of lysosomes and release of their enzymes (Fikrig, 1968). Gold treatment of RA patients suppresses the blastogenic response of human lymphocytes cultured from these patients. Gold sodium thiomalate inhibits DNA synthesis by sheep lymphocytes stimulated with PHA (Cahill, 1971). Gold also causes an increase in the number and size of multivesicular bodies in these stimulated lymphocytes possibly exerting its effect on lysosomal membranes in these bodies apparently in the region of the Golgi apparatus where the membranes are formed. This is of added interest since synovial fluid from RA patients, but not fluid from non RA patients, will transform autologous lymphocytes into blast cells _in vitro_ (Kinsella, 1973).

TABLE 1 Enzymes Inhibited by Gold

Enzyme	Source	Function	Conc Gold	Comment	Reference
Acid phosphatase β-glucuronid-ase	guinea pig peritoneal macrophage lysosome	hydrolase	1.25×10^{-3}M threshold	inhibit if Au incubation at 37°C not at 0°C	Persellin (1963, 1966)
Malic dehydrogenase	guinea pig peritoneal macrophage mitochondria				
Acid phosphatase Cathepsin β-glucuronid-ase	rheumatoid synovial fluid rabbit liver lysosomes	hydrolase	5×10^{-3}M 50% inhibition	non-competitive block -SH	Ennis (1968)
Acid hydrolase	rheumatoid synovial fluid	hydrolase	5×10^{-6}M 25% inhibiton		Lorber (1968b)
Acid phosphatase β-glucuronid-ase	rheumatoid synovial fluid extra- and intra-	hydrolase metabolism hyaluronic acid	6×10^{-5}M	higher conc. to inhibit extracellular enzyme	Paltemaa (1968a)
Cathepsin	cellular	proteolysis	9×10^{-6}M		

TABLE 1 continued

Enzyme	Source	Function	Conc Gold	Comment	Reference
Acid phosphatase β-glucuronid-ase	cell free rheumatoid synovial fluid	hydrolase	1.25x10^{-5}M	Au inhibits extracellular hydrolases	Lorber (1970)
Acid hydrolase	synovial fluid granulocytes		no effect 3x10^{-5}M		
Acid phosphatase Tryptophanyl tRNA synthetase	human epidermis	hydrolase			Penneys (1972)
Glucosamine-6-phosphate transamidase	rat granulation or liver tissue	synthesis of mucopolysaccharide of connective tissue	1.25x10^{-5}M threshold		Bollet (1960)
Glucosamine 6-phosphate synthetase	adjuvant arthritic rat liver and granulation tissue	synthesis of mucopolysaccharides	10^{-4}-10^{-5}M		Fujihara (1971)

TABLE 1 continued

Enzyme	Source	Function	Conc Gold	Comment	Reference
Glucosamidase	non-rheumatoid synovial fluid	hydrolysis of amides	5×10^{-4}M	non-competitive	Caygill (1965)
Elastase	human peripheral granulocyte lysosomes	elastic fibre degradation solubilization of basement membrane	5×10^{-4}M 5×10^{-3}M	inhibits elastolysis and esterolysis	Janoff (1970)
Pyruvate oxidase	pigeon brain				Thompson (1947)
Cholinesterase	horse serum	hydrolysis of acetyl choline	2×10^{-3}M 10-20% inhibition		Frommel (1944)
Protyrosinase Tyrosinase			6×10^{-8}M 5×10^{-7}M	activation	Bodine (1943)

Several other enzymes affected by gold are of particular interest in RA. Granulocyte elastase, another possibly destructive enzyme which is inhibited by gold probably has a role in elastic fibre degeneration in arteritis (sometimes seen in RA) and may also have a broader role in inflammation (Janoff, 1970). The proliferative stage of inflammation manifested by hyperplasia of the synovial membrane may be inhibited by gold through an action on enzymes involved in connective tissue synthesis. Inhibition of glucosamine-6-phosphate transamidase has been demonstrated (Bollet, 1960; Fujihara, 1971).

Gold as gold chloride, gold sodium thiomalate ($2.5\text{x}10^{-3}$M) or gold sodium thiosulfate ($3\text{x}10^{-4}$M) inhibits respiration in rat liver and kidney slices, (Block, 1945,1948) and is capable of uncoupling oxidative phosphorylation in respiring rat liver mitochondria (Whitehouse, 1963, 1964).

As part of their destructive action the released lysosomal enzymes cause denaturation of proteins some of which may serve as antigens stimulating further synthesis of IgG and perpetuating the inflammation. There is some evidence to suggest that gold may act to alter the manner in which protein is denatured. Bovine serum albumin denatured by heat in the presence of gold sodium thiomalate is more viscous than the protein denatured in the absence of gold. Suppression of intermolecular disulfide bond formation during denaturation is associated with a rise in viscosity. When urea is used as a denaturing agent, agents which react with sulfhydryl groups prevent the rise in viscosity normally associated with this denaturation. Gold prevents this rise in viscosity when urea is added. The concentration of gold required for these effects is $2x10^{-7}M$ (Gerber, 1964). Copper-catalyzed heat aggregation of human gamma globulin is inhibited by gold sodium thiomalate ($4x10^{-7}M$) (Gerber, 1971, 1974). This type of aggregation of gamma globulin may take place slowly in rheumatoid joints and may be interferred with by gold therapy. Gold sodium thiosulfate treatment of rats causes increased cross linkage of collagen protein producing a protein with decreased lability to denaturation and enzymatic degradation. This may be related to immune perpetuation of rheumatoid arthritis by reducing the amount of degraded protein, a possible antigen for continued synthesis of antibodies and continued inflammation (Adam, 1965, 1966, 1968a,b). In vitro incubation of collagen fibres with gold will produce similar effects provided gold is present in an ionic form (gold chloride), or if the gold is nonionic (gold sodium thiosulfate) if it is first oxidized. In vivo it is suggested that gold sodium thiosulfate first complexes with the protein and then slowly the thiosulfate is displaced, by protein side chain groups, leaving gold free to crosslink collagen fibres (Adam, 1968a). Gold thiomalate (10^{-3} to 10^{-6}M) does not inhibit the synthesis of collagen protein in granulation tissue so does not in this way interfere with the proliferative stage of rheumatoid arthritis (Aalto, 1972). The increased degradation of collagen caused by parathormone is prevented by gold (Burkhardt, 1971). Circulating levels of other proteins are reported to be altered by gold therapy. Successful gold therapy of rheumatoid arthritis results in decreased albumin, alpha-1 and alpha-2-glycoproteins and increased levels of beta-glycoprotein, while in patients in whom gold therapy caused no improvement alpha-1-glycoproteins are increased and beta-glycoproteins are decreased (Bernacka, 1966). This is of interest when the transport role of these proteins is remembered. Elevated levels of five glycoproteins in rheumatoid arthritis have been reported to be lowered to normal levels by gold therapy (Rau, 1968).

A polypeptide has been isolated which may function to initiate the transition from the exudative to the reparative phase of inflammation by inducing hypermetabolism in synovial cells. This possible point of interference with the disease process is susceptible to inhibition by several antirheumatic drugs including cortisol, phenylbutazone, and acetylsalicylic acid but not gold (Castor, 1972).

The suggestion that improvement in rheumatoid arthritis can occur through enhancement of blood fibrinolysis and subsequent removal of fibrin from inflamed tissue has been made. Gold sodium thiosulfate therapy in rheumatoid arthritis does lead to enhanced blood fibrinolytic activity and concomitant reduction in alpha-2-globulin and plasma fibrinogen levels (Andersen, 1968). The decrease in alpha-2-globulin may be the indirect cause of accelerated fibrinolytic activity by lowering antiplasmin effects.

The amino acid tryptophan has been postulated to mimic certain polypeptides which protect tissues from inflammatory insults. In order to exert this protective effect the peptides must be present in non-protein bound form in the plasma. Several antirheumatic drugs, including gold, are able to displace tryptophan from its binding sites in vitro. If displacing tryptophan from its binding sites is necessary for the action of these drugs the bound level of the drugs may be responsible for the antirheumatic action, quite the opposite to usual considerations that free drug is the active form (McArthur, 1971a,b).

Gold therapy has been shown to alter the serum level of some essential and non-essential trace metals. Molybdenum, manganese, tin, barium and cesium levels which are elevated in rheumatoid arthritis were reduced below the levels normally found in healthy volunteers. Copper levels which were elevated in rheumatoid arthritis were further elevated by chrysotherapy. Zinc and iron, which are lowered in rheumatoid arthritis, were not affected by gold. Essential trace metals, through their role as cofactors, may be of significance to enzyme activity. Excesses of nonessential trace metals may act as enzyme inhibitors blocking some metabolic function and producing disease. Gold could act to cure this metabolic lesion by displacing the nonessential metal (Niedermeier, 1971a,b).

A new theory of inflammation suggests that prostaglandins play a major role in the process. Debye (1973) showed that gold chloride or sodium aurothiopropanol sulfonate (10^{-4} to 10^{-5}M) inhibit the in vitro synthesis of prostaglandins E-2 and F-2-alpha.

Because of the antiarthritic effect of corticosteroids it has been suggested that gold may act via stimulation of the pituitary-adrenal axis. Mills (1963) and Sawyer (1964) showed in studies with rats that gold increases reduction of cortisone at the delta^{4}-3-keto position, the metabolic reaction taking place in the reticulo-endothelial (RE) cells of the liver and the adrenal. Heavy metals are in some cases removed via the RES system resulting in powerful stimulation of the system which could explain the enhanced metabolism of steroids. This study revealed no effect on adrenal synthesis of corticosteroids per unit weight of adrenal tissue; however there was an increase in adrenal weight which would result in an overall increase in corticoid production. The increased metabolism combined with increased production may balance out any net effect . Wegg (1955) showed that gold stimulates adrenocorticotrophic hormone (ACTH) release by the pituitary, measured by depletion of adrenal ascorbic acid. Vykydal (1956) showed gold as well as bismuth and copper increased the excretion of corticosteroids by rats. Schaposnik (1953) studying rheumatoid arthritis patients receiving gold therapy found increased excretion of 17-ketosteroids in males and irregular effects in females, while Roskam (1953) found increased excretion of 17-ketosteroids in all gold treated patients. Bruce (1952) was able to find no marked effect on 17-ketosteroid excretion during or after gold therapy compared with pretreatment excretion. Estimates of urinary 17-ketosteroids are however not a good indication of adrenal cortex activity because the level is influenced by secretion of 17-ketosteroid by the gonads and is also influenced by kidney and liver function. While our study was in progress Kirchheiner (1973) reported determination of plasma, plasma ultrafiltrate and tissue levels of cortisol in rheumatoid arthritis patients treated with gold. Tissue levels of cortisol dropped significantly (51 ng/g to 20 ng/g) when pretreatment levels were compared to postreatment levels. Plasma and plasma ultrafiltrate levels were not affected. Reduction in tissue cortisol level was not correlated with rate of improvement of rheumatoid arthritis . The change in tissue cortisol may have been due to gold treatment, due to improvement in rheumatoid arthritis, or due to some other factors. Gronbaek (1960) found elevated Na/K ratios in sweat of rheumatoid arthritis patients after gold therapy compared to the same patients prior to gold therapy. This was taken as evidence against gold stimulation of the adrenal cortex and may even indicate blockade of the adrenal cortex since adrenal cortex stimulation is associated with decreased Na/K in sweat and insufficiency of adrenal cortex function with increased Na/K. Changes in sweat flow or in body temperature did not occur with gold therapy so could not explain the altered Na/K ratio.

B. THE ROLE OF THE ADRENAL CORTEX IN RHEUMATOID ARTHRITIS

Function of the hypothalamic-pituitary-adrenal axis (H-P-A) is not grossly altered by rheumatoid arthritis (Hill, 1966); however subtle changes do occur, the significance of which is difficult to establish. Some authors feel the changes seen in rheumatoid arthritis are not unique to this disease but are secondary to any chronic disease. The response of the adrenal cortex to adrenocorticotrophin (ACTH) is normal when measured by circulating levels of 17-hydroxycorticosteroids (the chief steroid of this group is cortisol) in adults or children with rheumatoid arthritis (Birke, 1958; Hill, 1961; Hughes, 1962). In children with RA, ACTH caused increased plasma levels of corticosterone with a normal cortisol response suggesting shunting of more precursors into the mineralocorticoid side of the steroid biosynthetic pathway (Figure 1). ACTH stimulation of RA patients results in a lower than normal excretion of 17-hydroxycorticosteroids, to a lesser extent decreased excretion of 17-ketosteroids, and increased excretion of aldosterone; however a similar response was seen in patients with non-rheumatoid chronic diseases (Birke, 1958; Hill, 1966).

Adrenal Steroidogenesis

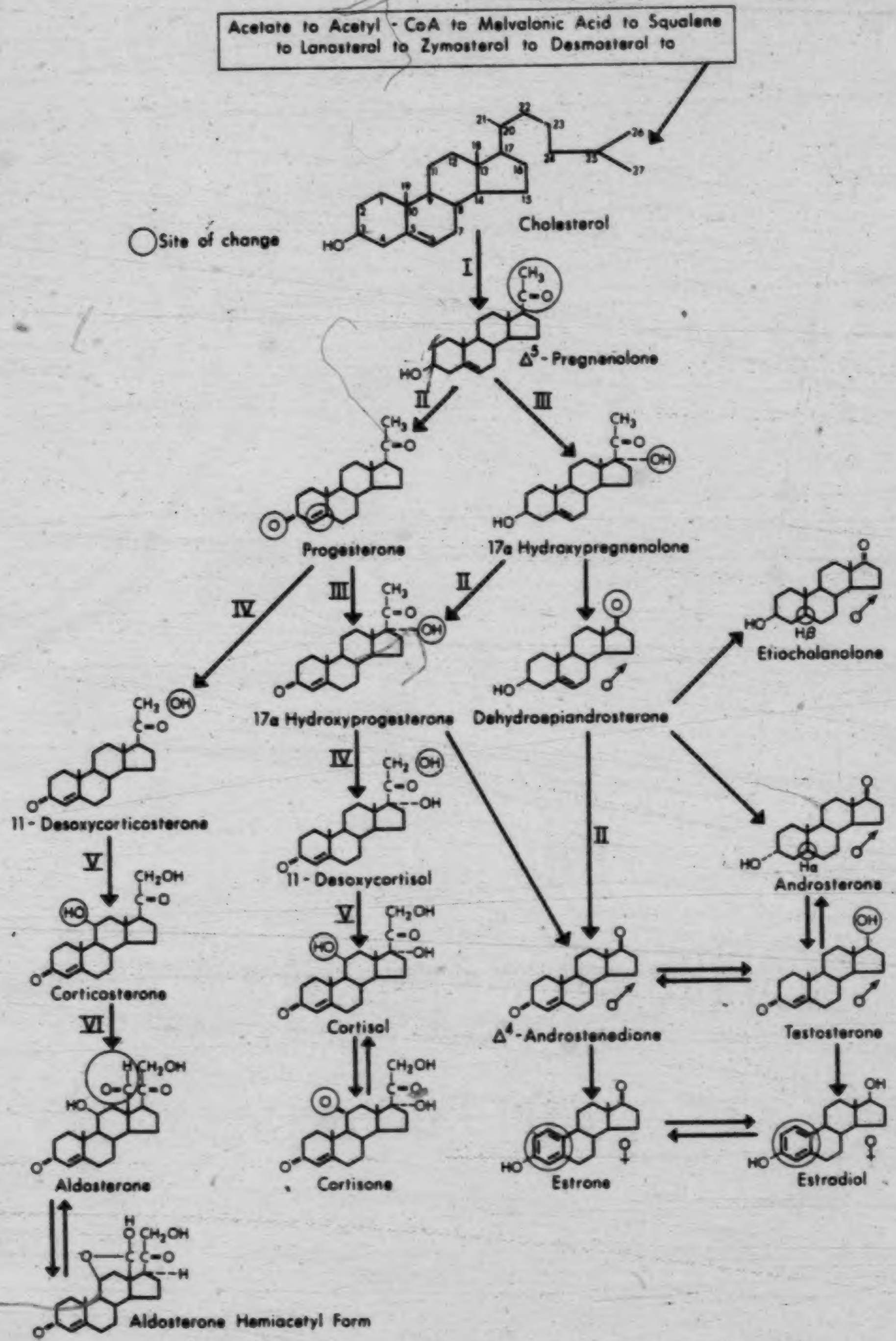

Acetate to Acetyl - CoA to Melvalonic Acid to Squalene to Lanosterol to Zymosterol to Desmosterol to
Cholesterol
Site of change
I
Δ5 - Pregnenolone
II
III
Progesterone
17a Hydroxypregnenolone
IV
III
II
17a Hydroxyprogesterone
Dehydroepiandrosterone
Etiocholanolone
11 - Desoxycorticosterone
IV
11 - Desoxycortisol
II
Androsterone
V
V
Corticosterone
Cortisol
Δ4 - Androstenedione
Testosterone
VI
Aldosterone
Cortisone
Estrone
Estradiol
Aldosterone Hemiacetyl Form

FIGURE 1 Adrenal Steroidogenesis. The following lists the enzymes involved.

I. Desmolase (mitochondrial fraction of cell).
II. 3-β-Dehydrogenase and 3-Ketoisomerase (microsomal fraction).
III. 17-Hydroxylase (soluble fraction).
IV. 21-Hydroxylase (soluble fraction).
V. 11-β-Hydroxylase (mitochondria).
VI. C_{18}-Hydroxylase.

(after Dillon, 1973)

Under basal conditions excretion of 17-ketosteroids, cortisol, cortisone, and various unconjugated metabolites (6-beta-hydroxycortisol, 20-hydroxycortisol, 20-hydroxycortisone) or conjugated metabolites (tetrahydrocortisone, allotetrahydrocortisol, cortols, cortolones, 11-beta-hydroxyetiocholanolone, 11-beta-hydroxyandrosterone, 11-oxoetiocholanolone) is normal in some studies (Bailey, 1966; Gray, 1965; Hill, 1959); and subnormal in at least a portion of RA patients in other studies (Birke, 1958; Hill, 1959; Hill, 1966; Pal,1967) (Figure 2). The normal diurnal pattern of highest excretion of 17-hydroxycorticosteroids in the morning is smoothed out in RA (Hill, 1959). Cortisol levels in blood of rheumatoid patients are normal (Birke, 1958; Gray, 1965; Hughes, 1962; Hvidberg, 1968; Winter, 1966), while corticosterone levels are elevated in children (Hughes, 1962). The binding of cortisol by transcortin and albumin is also normal in RA (DeMoor, 1962; Winter, 1966). Synovial fluid levels of cortisol are low compared to plasma but transcortin and albumin levels are also lower resulting in similar levels of unbound cortisol in plasma and in synovial fluid (Winter, 1966). Skin levels of cortisol in RA and other collagen diseases tend to be higher than in non-collagen diseases (Hvidberg, 1968).

Both hepatic and extrahepatic metabolism of corticosteroids (Figure 2) is normal in rheumatoid arthritis (Bailey, 1966; Birke, 1958; Gray, 1965); except for plasma levels of cortisol 21-acetate which are elevated (Margraf, 1972). In most assays this metabolite deacetylates during isolation and is measured as nonmetabolized cortisol. Because this metabolite is more strongly protein bound than cortisol itself, this may reduce the amount of measured cortisol in the active form. Secretion rates and turnover of cortisol are normal in RA (Gray, 1965; Hill, 1966;Peterson, 1955).

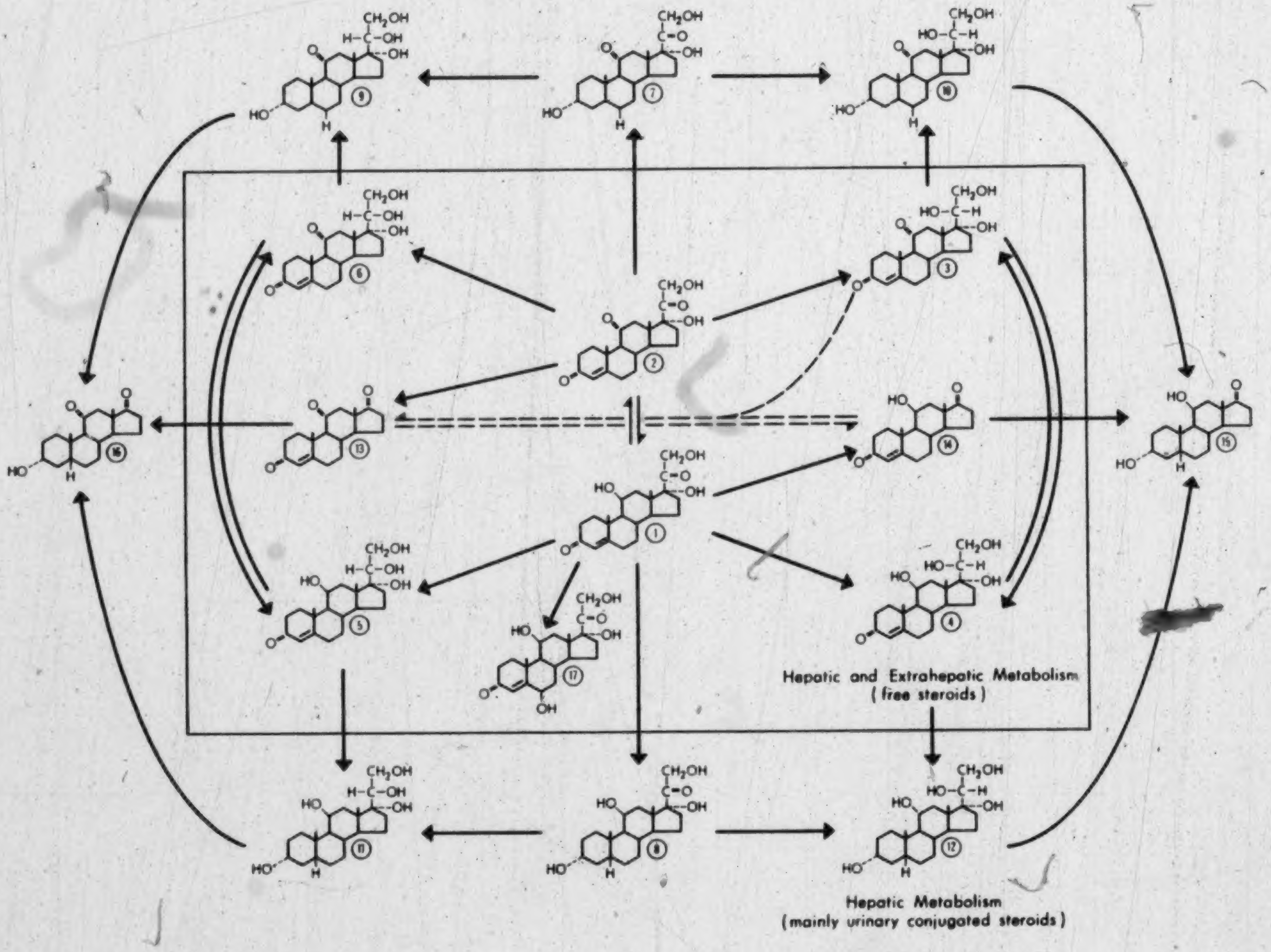

Hepatic and Extrahepatic Metabolism
(free steroids)
Hepatic Metabolism
(mainly urinary conjugated steroids)

FIGURE 2 Hepatic and extrahepatic metabolism of cortisol: (1) 4-pregnene-11β, 17α, 21-triol-3, 20-dione (cortisol); (2) 4-pregnene-17α, 21-diol-3, 11, 20-trione (cortisone); (3) 4-pregnene-17α, 20β, 21-triol-3, 11-dione (substance U of Reichstein); (4) 4-pregnene-11β, 17α, 20β, 21-tetrol-3-one (substance E of Reichstein); (5) 4-pregnene-11β, 17α, 20α, 21-tetrol-3-one (20-epi substance E of Reichstein); (6) 4-pregnene-17α, 20α, 21-triol-3, 11-dione (20-epi substance U of Reichstein); (7) pregnane-3α, 17α, 21-triol-11, 20-dione (tetrahydrocortisone); (8) pregnane-3α, 11β, 17α, 21-tetrol-20-one (tetrahydrocortisol); (9) pregnane-3α, 17α, 20α, 21-tetrol-11-one (α-cortolone); (10) pregnane-3α, 17α, 20β, 21-tetrol-11-one (β-cortolone); (11) pregnane-3α, 11β, 17α, 20α, 21-pentol (α-cortol); (12) pregnane-3α, 11β, 17α, 20β, 21-pentol (β-cortol); (13) 4-androstene-3, 11, 17-trione (androsterone); (14) 4-androstene-11β-ol-3,17-dione (11β-OH-androstenedione); (15) etiocholane-3α, 11β-diol-17-one (11β-OH-etiocholanolone); (16) etiocholane-3α-ol-11, 17-dione (11-keto-etiocholanolone); and (17) 4-pregnene-6β, 11β, 17α, 21-tetrol-3, 20-dione (6β-OH-cortisol).

(after Berliner, 1961)

Relative changes in biosynthetic pathways (Figure 1) have been reported in RA. These changes include decreases in 11-beta- and 17-alpha-hydroxylases, 11-beta-dehyrogenases and 5-alpha-reductases resulting in slight increases in corticosterone and 11-deoxycorticosterone, increase in cortisol relative to cortisone, and relative increase in 5-beta-steroids such as etiocholanolone (Hill, 1966; Mulder,1970).

Choice of adequate controls in these type of studies is difficult since many factors such as sex, age, state of health, degree of activity, drug therapy, and time of sample collection have to be considered. This is reflected in the studies reported above by the various ways in which controls were used. Hill (1959) attempted to be very thorough in his choice of controls. His study was confined to male patients in six categories: young healthy ambulatory males, older (closer match to the disease groups) healthy ambulatory males, ambulatory males with active RA, hospitalized males with nonrheumatic chronic disease, and finally hospitalized males with no disease. Hospitaliztion per se did not alter corticoid excretion; both ambulatory and hospitalized RA patients lost diurnal variation in corticoid excretion. The chronic disease group fell between normal groups and RA groups in corticoid excretion and although morning corticoid excretion was significantly lower in RA groups than in normal groups, it was not significantly different from chronic disease groups. In this study drug therapy was not controlled: two of twelve hospitalized RA patients and three of eleven ambulatory RA patients were on salicylates, while four of eleven ambulatory RA patients were on gold therapy. Margraf (1972) used outpatient controls recovering from simple fractures or minor wounds. The RA groups and control groups were each subdivided into three groups: those on acetylsalicylic acid, on prednisolone, and on no drugs. Similar amounts of cortisol 21-acetate were found in all three subgroups and the difference between control and RA was seen in each subgroup. Hughes (1962) used hospitalized children as controls but did not consider drug therapy. Winter (1966) used nonrheumatoid controls; Pal (1967) ambulatory controls; Bailey (1966), Hvidberg (1968), and Birke (1958) did not describe their controls while Gray (1965) used normal values obtained from the literature. Pal (1967) and Hvidberg (1968) excluded or separated patients on steroids or ACTH; the latter also separated RA patients receiving indomethacin from other RA patients. Bailey (1966) and Gray (1965) had some patients on acetylsalicylic acid, phenylbutazone or hydroxychloroquine. Birke (1958), Hill (1966) and Winter (1966) made no mention of drug therapy.

C. EFFECT OF SELECTED DRUGS ON ADRENOCORTICAL FUNCTION

Possible alteration of adrenal cortex function is not unique to gold. Studies of the relationship of other anti-arthritic drugs and the hypothalamo-pituitary-adrenal axis have been reported in the literature. Secondly, some of the patients of this study were taking drugs other than anti-arthritic drugs; therefore a search of the literature for reports of actions of these drugs on adrenal cortex was made. It must be kept in mind that toxic doses of any drug will cause alteration in hypothalamo-pituitary-adrenal function; this is a non-specific stress reaction, not a direct effect of the drug.

It is well known that systemic steroid treatment of RA results in suppression of adrenal cortex function (Hansten, 1971). Several studies of long term (2 months to 15 years) RA therapy with prednisone, prednisolone or methylprednisolone (total dose 3 to 29 g) showed subnormal response of the adrenal cortex to ACTH during intermittent or continuous therapy. Also responses were subnormal in a significant portion of patients being withdrawn from steroid therapy (Goth, 1966; Jakobson, 1968; Daly, 1967). Pituitary response to metyrapone or lysine-vasopressin was suppressed earlier than adrenal cortex response (Goth, 1966; Jasani, 1967; Jakobson, 1968). Intra-articular steroid can have a systemic suppressive effect on H-P-A function. In a study using dogs treated with triamcinolone diacetate (intra-articularly), response of plasma cortisol to insulin stress was subnormal (Wellington, 1970). The suppression of H-P-A was not fully recovered till 12 weeks after injection while the anti-inflammatory effect in the joint, measured by challenge with urate crystal injection, lasted only 2.5 weeks. Systemic effects of other steroids given intra-articulary would depend on the solubility of the particular steroid.

Kelley (1961) has reviewed the conflicting research on salicylate effects on adrenocortical function concluding that large doses of salicylates do activate the adrenal cortex via pituitary stimulation. He postulated that enhanced metabolism of corticosteroids coupled with enhanced biosynthesis may partially explain the opposing results. Maintenance of elevated plasma corticosteroid levels is not necessary for the anti-arthritic effect of salicylate. Subsequent research has verified that enhanced metabolism of corticosterids does occur via 3-alpha-dehydrogenation of dihydrocortisol, when salicylates are added in vitro to rat liver homogenates (Sedlak, 1972a). Corticosteroid glucuronide formation is not enhanced by salicylates (Sedlak, 1972b). Maickel (1965) reported increased unbound corticosterone levels in salicylate treated rats which is supported by the In vitro findings of Stenlake (1971) that large doses of acetylsalicylic acid decrease cortisol binding, likely affecting its binding to albumin. In vivo in guinea pigs Jansen (1971) could not find changes in levels of bound or unbound cortisol in plasma, skin cortisol, plasma half life of exogenous cortisol or its volume of distribution. Salicylates inhibit membrane uptake of cortisol by isolated liver lysosomes thus abolishing the membrane stabilizing effect of cortisol (Lewis, 1971).

Indomethacin does not alter plasma cortisol (total or unbound), skin cortisol, or the half life or volume of distribution of exogenous cortisol in guinea pigs (Jansen, 1971, 1972). In rats unbound corticosterone was increased by indomethacin (Maickel, 1965); however in humans, binding of cortisol is unaffected by indomethacin (Stenlake 1968, 1971).

Chloroquine was reported to elevate plasma 17-hydroxycorticoids in disseminated lupus erythematosis patients (Roskam, 1955). This finding was not confirmed in guinea pigs treated with chloroquine (Jansen, 1972). Plasma cortisol (total and unbound), skin cortisol, and cortisol half life and volume of distribution were unaffected by chloroquine administration (70 mg/kg/d x 3 d). In a long term experiment using rats dosed with chloroquine (30 mg/kg/d x 24 wk) morphologic changes in the zona fasiculata of the adrenal cortex (the layer of the adrenal producing glucocorticoids) suggestive of adrenal cortex activation were correlated with maximum accumulation of chloroquine in the adrenal (Grundman, 1973).

Phenylbutazone (48 mg/kg/d x 3 d) treatment of guinea pigs increased the volume of distribution of exogenous cortisol but did not alter skin or plasma (total and unbound) levels of cortisol (Jansen, 1971). Similar observations of increased volume of distribution of corticosterone in rats had been reported by Maickel (1966). Higher doses of phenylbutazone (200 mg/kg/d x 3 d) increased total plasma cortisol and volume of distribution of cortisol while skin levels, unbound cortisol in plasma, and half life of cortisol were unaffected (Jansen, 1972). *In vitro* and *in vivo* phenylbutazone does not alter plasma binding of cortisol (Stenlake, 1968, 1971). *In vitro* addition of large doses (four times therapeutic) of oxyphenbutazone to plasma decreased unbound cortisol due to increased albumin binding of cortisol (Stenlake, 1971). In rats, phenylbutazone has been reported to act on the CNS to stimulate ACTH secretion (Gold, 1967).

Flufenamic acid, an analgesic of limited use in RA, was shown by Maickel (1965) to increase unbound corticosterone levels in rats. Ibufenac, another analgesic of limited value in RA, has no demonstrable effects on cortisol binding to human plasma (Stenlake, 1971) .

Drugs, other than anti-arthritic coumpounds were being used by some patients in this study. A search of the literature revealed that some of them may alter adrenocortical function. Alcohol, a drug commonly used in society, produced marked elevation of plasma cortisol in man when blood alcohol levels exceeded 100 mg % (Dillon, 1973). At lower blood alcohol levels, plasma levels of cortisol are slightly decreased and urinary excretion of cortisol is elevated probably because of the diuretic effect of alcohol. Resting ACTH secretion in man is decreased by alcohol (Gold, 1967). In alcoholics, ingestion of alcohol increased cortisol levels when compared to cortisol levels in non-drinking periods and compared to non-alcoholics (Stokes, 1973). Caffeine, another commonly ingested drug, increases plasma cortisol levels in man and in dogs (Avogaro, 1973; Bellet, 1969).

Estrogens elevate the circulating level of transcortin (Lipsett, 1972; Bulbrook, 1973; Gaunt, 1968). The body compensates for the resultant increased binding of cortisol with increased secretion, thus total plasma cortisol is also elevated (Bulbrook, 1973; Lindholm, 1973; Hvidberg, 1968). It is commonly thought that the opposing effects of increased total cortisol and increased binding will balance out preventing excess levels of unbound cortisol and hence preventing symptoms of hypercorticoidism. This is supported by the usual observation of decreased urinary excretion of cortisol and corticoids, excretion of unmetabolized cortisol being a function of cortisol in the blood (Bulbrook, 1969). In addition, skin levels of cortisol, which would likely be a function of unbound cortisol levels, are unchanged by estrogen treatment (Hvidberg, 1968). Lindholm (1973), however, found elevated unbound plasma cortisol in women treated with estrogen/progestagen oral contraceptives. Estrogen has been reported to increase ACTH secretion in both man and rats (Gold, 1967; Kitay, 1968). In rats estrogens inhibit intraadrenal reduction of corticosterone to the inactive metabolite 3-beta-5-allotetrahydrocortisone, thus estrogens elevate corticosterone secretion (Kitay, 1968). Hepatic metabolism of corticoids by ring A reduction is increased by estrogens resulting in increased corticoid clearance (Kitay, 1968; Colby, 1973) while reduction at the C-20 position to form beta-cortol is decreased (Gillie, 1973).

Phenobarbital, because of its enzyme inducing properties, might be expected to alter steroid biosynthesis and metabolism. Steroid hydroxylases (17,18 and 19) are not affected by phenobarbital (Sheppard, 1967) therefore biosynthesis is not altered. Metabolism of cortisol by 6-beta-hydroxylation, in man but not in guinea pig, and by 2-alpha-hydroxylation in guinea pig, is enhanced by phenobarbital (Lipsett, 1972). Conflicting evidence has been presented as to whether phenobarbital decreases or has no effect on ACTH secretion (Gaunt, 1963, 1968; Gold 1967).

No clinically significant effect of glutethimide on adrenocortical function was reported by Gaunt (1968) or Chart (1968), while a transient inhibition of corticosteroid secretion in rats was reported by Daniel-Severs (1973) possibly due to inhibition of biosynthesis between the pregnenolone to corticosterone conversion step (Johnston, 1968). This inhibitory effect required larger than therapeutic doses of glutethimide (Johnston, 1968).

Thyroxine in large doses increased the secretion of corticosteroids (Gold, 1967) and also altered the 17-ketosteroid biosynthetic pathway in favor of greater concentrations of 5-alpha-steroids relative to 5-beta-steroids (Gaunt, 1968). Turnover of cortisol in man is enhanced by elevated thyroxine levels due to increased hepatic metabolism by increased reduction of the 4-5 double bond and increased 6-beta-hydroxylation (Gold, 1967; Lipsett, 1972). Thyroxine does not alter transcortin levels.

Search of the literature revealed no reports of alteration of adrenocortical function by any of the following drugs which some of the patients were taking: amitriptyline, chlordiazepoxide, chlorpheniramine, codeine, dextropropoxyphene, diazepam, ferrous sulfate, methotrexate, orphenadrine, primidone or propantheline.

D. APPROACHES TO THE STUDY OF CORTISOL PROTEIN BINDING

(i) THEORETICAL CONSIDERATIONS

Cortisol circulating in plasma is predominantly protein bound. It is bound chiefly to two proteins: a specific carrier protein of the alpha-1-globulin type, transcortin, has a high binding affinity for cortisol but a low capacity, while albumin has a low affinity but a large capacity. Protein bound cortisol is generally considered to be inactive (Beisel, 1964a). In the protein bound form cortisol is readily available by dissociation, but protected from metabolism, and excretion by filtration. Any agents which cause a change in protein binding of cortisol will alter the amount of unbound active cortisol as well as alter the rate of turnover of cortisol.

Binding properties of a protein can be described by its affinity (K) and its capacity (P), or if the concentration and molecular weight of the protein is known, by the number of binding sites (N) of a given type. Affinity is the strength with which a protein binds a particular ligand and is a measure of the quality of the protein with respect to binding. On the other hand capacity is a measure of the quantity of binding protein. In plasma transcortin is present at concentrations of about 7 mg% and cannot be isolated for binding studies without large amounts of sample (Turner, 1971). Furthermore isolated transcortin may not be identical to native transcortin. Since albumin and transcortin are the only important cortisol binding proteins in plasma, it is possible to study the binding properties of transcortin and albumin in plasma itself.

Formulae have been worked out to describe the binding of a ligand to two distinct binding sites (Edsall, 1958) which are applicable to binding of a ligand to two proteins each containing a single type of binding site for the ligand. Isolated transcortin has only one binding site for cortisol while albumin has two binding sites, as determined using progesterone (Burton, 1972). Symbols have been altered slightly from those used by Edsall, since in plasma concentrations of each protein are not known and binding capacities, not number of binding sites, are determined.

From the law of mass action $B + A \rightleftharpoons BA$

and Kassoc = [BA]/([B][A]) = 1/Kdissoc (1)

where

B = concentration of unoccupied binding species (in the simplest case binding activity = concentration, ie 1 binding site per molecule of B).

A = concentration of unbound ligand

BA =

concentration of ligand bound to binding species

$V = [BA]/[BA + A]$ (by definition)

$V = K[B][A]/(K[B][A] + [B])$

(substitute for BA as defined in 1).

$V = K[A]/([A] + 1)$ (2)

where

V = average number of moles of A bound for all molecules of B and BA present, or the probability a molecule of B chosen at random has a molecule of A bound to it.

K = K association

$$V = \sum_{i=1}^{n} Vi = NV$$

where

V is redefined, assuming N equivalent and independent binding sites on one molecule of B, as total average number of occupied sites per molecule of B, and Vi is the probability the ith site is occupied (ie Vi is defined as V was defined in 2).

$$V = NVi = NK[A]/(K[A] + 1) \quad (3) \quad \text{(from 2)}$$

$$N = N1 + N2 + \ldots \; Nm$$

$$V = \sum_{i=1}^{m} \{ NiKi[A]/(Ki[A] + 1) \}$$

where

N = total number of binding sites, and there are m different sets of binding sites, first with N1 equivalent and independent binding sites with association constant K1, etc.

$$V/A = \sum_{i=1}^{m} NiKi/(Ki[A] + 1) \quad (4/A)$$

$$\lim_{A \longrightarrow 0} V/A = \sum_{i=1}^{m} NiKi$$

$$\lim_{A \longrightarrow \infty} V = \sum_{i=1}^{m} Ni = N$$

For 2 different sets of equivalent and independent binding sites $V = N1K1[A]/(K1[A] + 1) + N2K2[A]/(K2[A] + 1)$ (5)

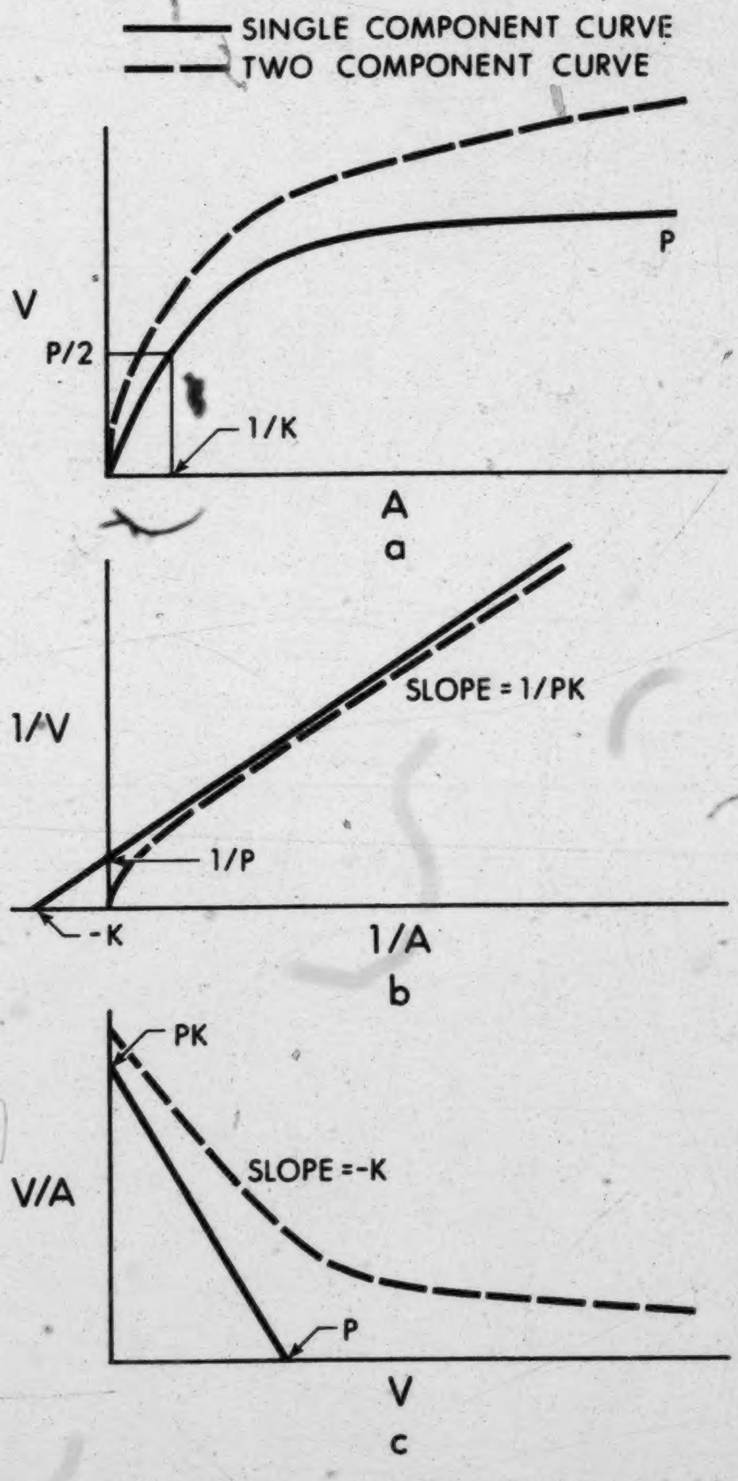
where the concentration of protein is not known equation 5 becomes $V = P1K1[A]/(K1[A] + 1) + P2K2[A]/(K2[A] + 1)$ (6)
Assuming the sites are located on different proteins in a solution which contains the proteins in unknown concentrations, then N cannot be calculated but binding

capacity of the solution can be calculated. Binding capacity is defined by symbols P1 and P2 and has units of quantity of ligand bound per given volume of protein solution. V has units of concentration but is no longer expressed as molar concentration. For cortisol binding to plasma P1 and K1 will be defined as transcortin binding capacity and affinity (association constant) and P2 and K2 will be defined as albumin binding capacity and affinity.

Binding data may be plotted in several ways. The simplest plot is that of bound (V) versus unbound (A). This plot (Figure 3a) is curved for a one component binding system. The change in curvature for two component binding curves is difficult to assess. This method of handling data has been used later in the mathematical resolution of two component binding data.

The Lineweaver-Burk or reciprocal plot method which is linear for a single binding component, (Figure 3b) is criticized because undue weight is given to values at low concentrations, values which are probably determined with least accuracy (Wilkinson, 1961). This method gives linear plots for one component binding data therefore P and K can be determined easily; however, for two component curves the slight curvature may be missed And estimates of P1 and K1 would be inaccurate.

The Scatchard plot (Figure 3c) of V/A versus V is linear for a single component binding curve and is curvilinear for two component binding data. There is however no equation describing a two component binding curve in terms of this type of plot.



EQUATION OF SINGLE COMPONENT BINDING CURVE

$$V = \frac{PKA}{KA+1}$$

$$\frac{1}{V} = \frac{KA+1}{PKA}$$

$$\frac{1}{V} = \frac{1}{P} + \frac{1}{PK} \cdot \frac{1}{A}$$

$$VKA + V = PKA$$

$$VK + \frac{V}{A} = PK$$

$$\frac{V}{A} = PK - VK$$

FIGURE 3 Graphical Representation of Binding Data.

A two component binding curve does not readily lend itself to resolution into individual component curves. In order to obtain estimates of transcortin affinity (K1) and capacity (P1) and albumin affinity (K2) and capacity (P2), several approaches have been used.

Hart (1967) showed that binding data could be resolved by solution of a set of simultaneous equations. In the case of two component binding data four estimates of bound and unbound are required to solve the set of four simultaneous equations. Similar equations have been worked out to define the systems used in this study.

Rearranging equation (6)

$$V = A\left\{\frac{P1K1}{K1A+1} + \frac{P2K2}{K2A+1}\right\}$$

Right side to common denominator

$$V = A\ \frac{P1K1(1 + K2A) + P2K2(1 + K1A)}{(1 + K1A)\qquad(1 + K2A)}$$

Cross multiply

$$V(1 + K1A)(1 + K2A) = A[P1K1(1 + K2A) +$$

$$P2K2(1 + K1A)]$$

Expand

$$V + VK1A + VK2A + VK1K2A^2 = P1K1A + P1K1K2A^2$$

$$+ P2K2A + K1K2P2A^2$$

Collect terms

$$V = K1A(P1 - V) + K2A(P2 - V) + K1K2A^2(P1 +$$

$P2 - V)$

Expand right side

$V = K1P1A - K1VA + K2P2A - K2VA + K1K2P1A^2$

$+ K1K2P2A^2 - K1K2VA^2$

Collect unknown terms (i.e. K1, K2, P1, P2)

$V = A(K1P1 + K2P2) - VA(K1 + K2) + A^2$

$(K1K2P1 + K1K2P2) - VA^2(K1K2)$

Divide by V

$1 = (A/V)\ (K1P1 + K2P2) - A(K1 + K2) +$

$(A^2/V)(K1K2P1 + K1K2P2) - A^2(K1K2)$ (7)

Unknown terms (in parentheses) are defined

$C = K1P1 + K2P2$ (8)

$D = K1 + K2$ (9)

$E = K1K2P1 + K1K2P2$ (10)

$F = K1K2$ (11)

Equation (7) can be rewritten

$1 = CA/V - DA + EA^2/V - FA^2$ (12)

The coefficients C to F can be calculated by solving a set of 4 simultaneous equations obtained by substituting A and V from 4 sets of data into equation 12.

Equation 9 and 11 are terms of the quadratic

$(Z - K1)(Z - K2) = 0$

which expands into

$Z^2 - (K1 + K2)Z + K1K2 = 0$

which can also be written

$Z^2 - DZ + F = 0$ (13)

The values of K1 and K2 can be obtained by solving the quadratic. The values of P1 and P2 can be obtained by solving simultaneous equations 8 and 10 after obtaining the values of C, E, K1 and K2.

A FOCAL program (Appendix I) which is run on a PDP-1105 computer (Digital Equipment Corporation, Maynard, Massachusetts) has been worked out to perform these calculations. When more than four sets of data are available binding parameters are calculated for all possible combinations of four and the mean and standard deviation of the calculated parameters are calculated. To illustrate the use of this program a set of test data (Figure 4) has been calculated by addition at constant unbound concentration the contribution of two single binding curves to obtain a composite binding curve. All points on a line passing through the origin of the plot V/A versus V are at constant unbound concentration (Rosenthal, 1967). The data was chosen such that it was similar to the experimental data Requiring resolution. Table 2 part 1 gives the true values of the parameters.

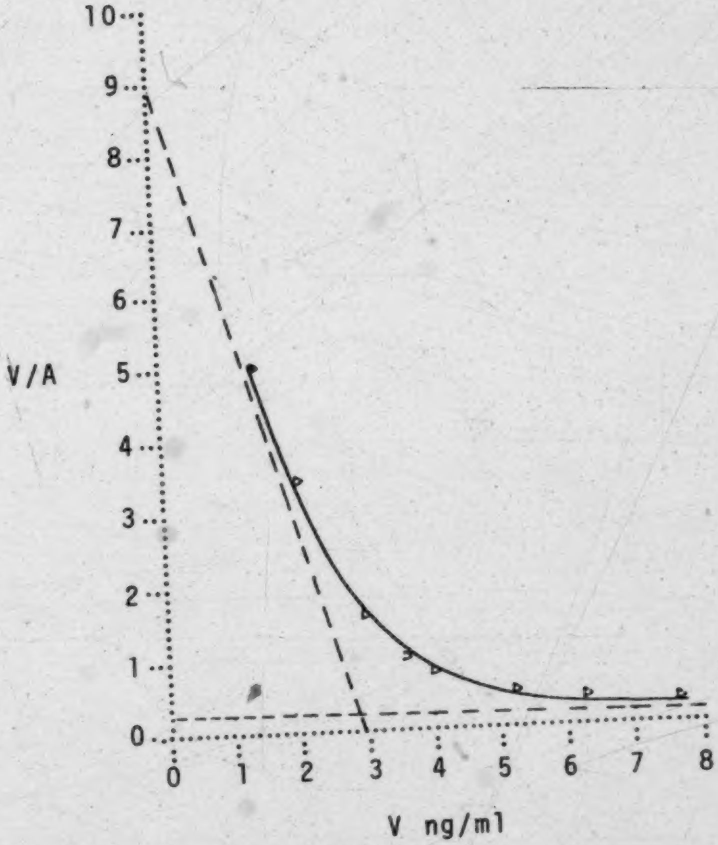


FIGURE 4 Simulated Protein Binding Data

—— composite curve
---- single component curves

P1=13.33 mcg%, K1=1.086 x 10^9 1/m, P2=111.11 mcg%, K2=3.62 x 10^6 1/m.

With highly accurate data this method of resolving a two component binding curve is also very accurate (Table 2 part 2). All possible data is utilized to calculate all parameters. Since there are eight sets of data all possible combinations of four is 8!/[4!(8-4)!] = 70. Introduction of small errors by rounding the data from 6 figures to 3 figures, results in errors in the estimated parameters K2 and P2 (Table 2 part 3) that is, this method of resolving data is very sensitive to small errors in the input data. In order to simulate error of the magnitude that might be expected in experimental data, error has been introduced randomly into V. This was done by calculation of V ± 5% then using a table of random numbers selecting either the upper or the lower limit. Now when the error in the input data is larger, negative values of K2 will sometimes occur in the calculations. Negative values of affinity are impossible and are not useful for determination of estimates of albumin affinity. As can be seen from Table 2 part 8 large errors occur in all estimated parameters when 5% error is introduced into V. If only positive values of K2 are accepted the estimated value of K2 is of the right magnitude (Table 2 part 4). If only positive values of P2 associated with a positive K2 are accepted P2 is biased upward by a factor of 10. It can be seen (Table 2 part 8 and Figure 5a) that high estimates of P1 occur when K2 is negative, whereas estimates of P1 are quite close to the true value when associated with positive K2. If only these latter estimates of P1 are accepted considerable data would not be used. If a literature value of K2 (2000 l/m DeMoor, 1964) is substituted in the calculations whenever negative K2's occur and only P1 associated with positive P2 are accepted, more data is used and the estimated P1 is closer to the true P1 (Table 2 part 4 and Figure 5b). The value of K2 substituted has little effect on the estimated P1 (Table 2 parts 4, 5, 6). The estimates of K1 which cluster about the true values of K1 are associated with initially positive K2, substituted K2 giving negative P2 and substituted K2 giving positive P2, so no selection of data on this basis can be made in calculation of K1 (Figure 5c). Low values of K1 are associated with substituted K2 where positive P2 results, and high values of K1 are associated with initially positive K2. By accepting all positive K1 the average is reasonably close to the true value of K1 (Table 2 part 4). The criteria utilized for accepting data sets for calculation of mean estimates of binding parameters were (1) all positive estimates of K1 were accepted, (2) all positive estimates of P1 associated with positive P2 were accepted, (3) all positive estimates of K2 where a literature value of K2 was not substituted were accepted, (4) all positive P2 values where a literature value of K2 was not substituted were accepted. This results in a fair estimate of K1 (42% high), A good estimate of P1 (within 10% of true value), and poor estimates of P2 and K2 (Table 2 part 4).

Admittedly this approach to acceptance of estimates of binding parameters is empirical and is not generally applicable. It is an attempt to deal with the problems which arise when a method which is accurate for theoretical data is applied to data which is somewhat in error, a situation that will arise when experimental data is used. One reason that poor estimates of P2 and K2 are obtained is that the data was obtained at relatively low concentrations of ligand compared to the binding capacity of albumin; errors in the data are magnified with respect to estimates of P2 and K2. The major purpose of this study was however to determine transcortin binding parameters as accurately as possible; information on P2 and K2 was considered of secondary importance. In future this method of resolving binding data is referred to as the mathematical method of resolution.

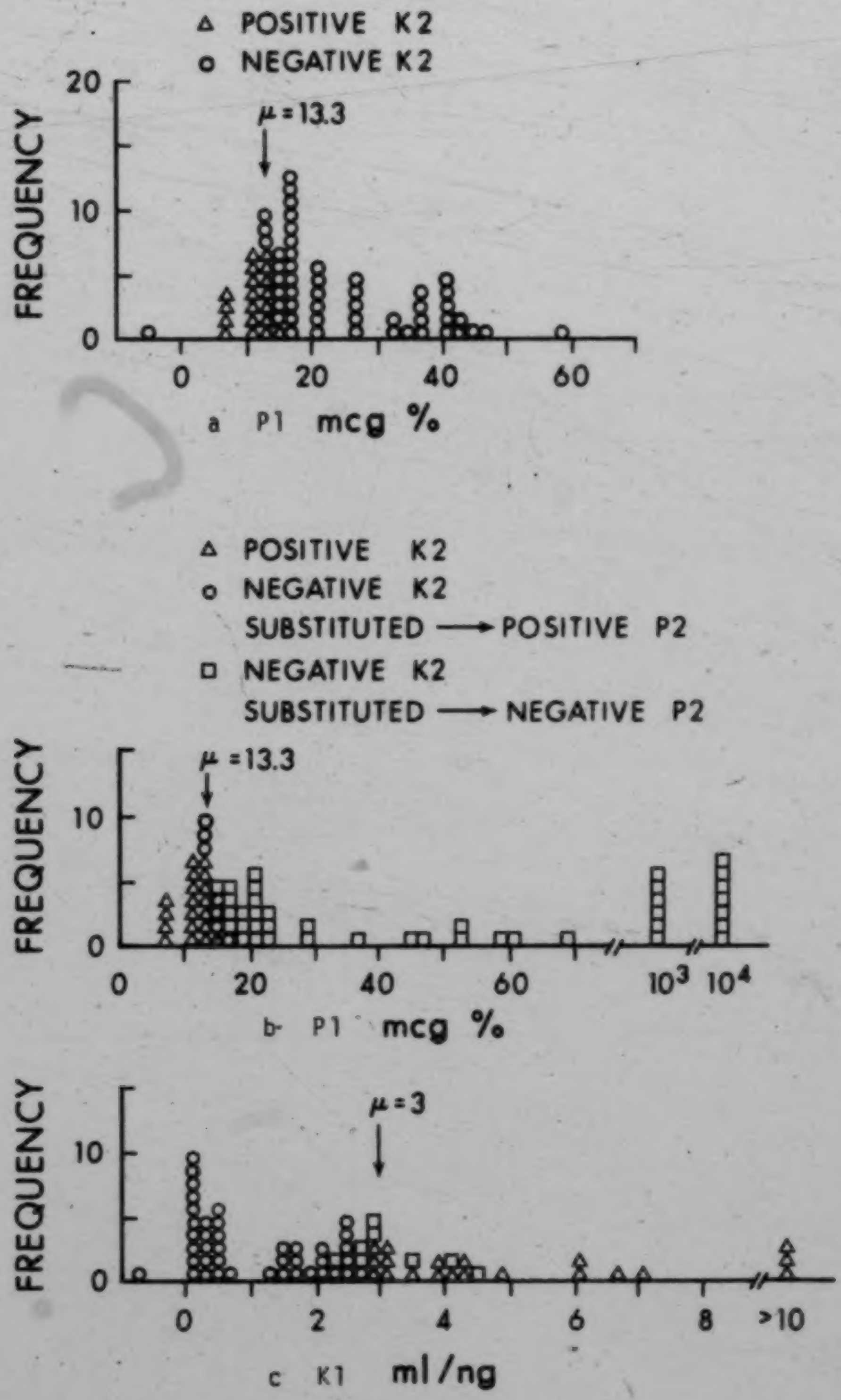


FIGURE 5 Estimated Parameters for Simulated Protein Binding Data

When more than four points are available another method to use all the data would be to fit a curve by the method of least squares to the experimental data then select four points on the curve for use in solving the equations. The drawback to this method is that the error in A is related to the error in V since V and T (which is total concentration) are measured and A is obtained by subtraction. There is no independent variable measured without error. Minimizing the sum of squares with respect to V is incorrect because both V and A are subject to error. An exact definition of the error of V and A is complex and the method of minimizing error about the fitted line is even more complex. Secondly the choice of four points to use in solving the equations is arbitrary, although if the fit is correct this will be of lesser importance (see small variation in predicted values of binding parameters Appendix I).

Another approach to resolving two component binding curves uses limits of equation 6.

$$V = \frac{P1K1A}{K1A + 1} + \frac{P2K2A}{K2A + 1} \quad (6)$$

As A approaches infinity K1A becomes very large and $KiA + 1 \simeq KiA$, so KiA in numerator and denominator cancel.

that is limit $(A \longrightarrow \infty)$ $V = P1 + P2$ (7)

If equation (6) is divided by A it becomes

$$\frac{V}{A} = \frac{P1K1}{K1A + 1} + \frac{P2K2}{K2A + 1} \quad (8)$$

As A approaches zero KiA becomes very small and $KiA + 1 \simeq 1$, thus the denominator disappears

$$\text{limit } (A \rightarrow 0)\ V/A = P1K1 + P2K2 \quad (9)$$

In the plot of V/A versus V the Y intercept for small values of V approximates the value of equation 9, while the X intercept for large values of V approximates the value of equation 7. When K1>> K2 the initial slope (small values of V) of the composite binding curve approximates -K1, while the final slope (large values of V) approximates -K2. If a straight line, $Y = HX + G$, is fitted through the initial portion of the curve the Y intercept, G, gives an estimate of P1K1 + P2K2, while the X intercept of a straight line, $Y = CX + D$, fitted through the final portion of the curve gives an estimate of P1 + P2.

$$K1 = -H \quad (10)$$

$$K2 = -C \quad (11)$$

$$P1K1 + P2K2 = G \quad (12)$$

$$P1 + P2 = -D/C \quad (13)$$

K1 and K2 are easily obtained while P1 and P2 can be obtained by solving the simultaneous equations 12 and 13.

In fitting straight lines to the two parts of the experimental curve, it is assumed that V is without error thus the sum of squares is minimized with respect to V/A. This is recognized as an approximation of the best fit since V is known to be subject to error as discussed earlier. This method of resolving binding data is less sensitive to errors in the data (compare Table 2 parts 9 and 12). The most important factor in getting good estimates of binding parameters by this method is the choice of points representing the extremes of the curve. If significant curvature exists in points used to fit the initial straight line K1 will be underestimated and P1 will be overestimated (Table 2 part 12 and 14). If significant curvature exists in points used to fit the latter part of the curve K2 will be overestimated and P2 underestimated (Table 2 part 14). Again because the concentration of bound ligand is small relative to P2, curvature in this part of the data almost always occurs so poor estimates of P2 and K2 are obtained. A program to do the calculations by this method, which will be referred to as the limit method, is given in Appendix I.

TABLE 2 SIMULATED PROTEIN BINDING DATA

Explanation of Table 2

Values given as mean, ± standard deviation, % error, n= number of sets of data used to calculate this mean and standard deviation.

1. True values.
2. Mathematical resolution; accepts: all positive K1, positive P1 where P2 also positive, positive K2, and positive P2 where literature value of K2 not substituted; literature value of K2 = 2000; data 6 figure accuracy.
3. As for 2 except data 3 figure accuracy which introduces 0.01 to 0.28% error in V.
4. As for 2 except 5% random error in V.
5. As for 4 except literature value K2 = 20000.
6. As for 4 except literature value K2 = 200000.
7. Mathematical resolution; set negative values K2 = 2000, reject set if any values of K1, P1 or P2 are negative, 5% random error in V.
8. Mathematical resolution; accept all data; 5% random error in V.

9-11. Limit method; data 6 figure accuracy.
12-14. Limit method; 5% random error in V.

	K1 x 10^9 1/m	P1 mcg%	K2 x 10^6 1/m	P2 mcg%
1.	1.086	13.3333	3.62	11.111
2.	1.086 ± 0 0% n=70	13.3334 ± 0.01 0.0005% n=70	3.61981 ± 0 0.0052% n=70	111.117 ± 0 0.0053% n=70
3.	1.09326 ± 0.051 0.67% n=70	13.342 ± 0.130 0.065% n=70	3.80867 ± 0.9 5.21% n=70	110.022 ± 19.5 9.8% n=70
4.	1.54229 ± 3.23 42.02% n=69	12.3118 ± 2.941 7.66% n=29	15.7355 ± 16.6 334.7% n=19	1291.51 ± 5003 1062.4% n=19
5.	As above	12.3119 ± 2.941 7.66% n=20	as above	as above

6.	As above	12.3128 ± 2.942 7.66% n=29	as above	as above
7.	3.1315 ± 4.553 188.4% n=29	as above	10.3102 ± 15.34 184.8% n=29	22710.4 ± 41616.3 20339% n=29
8.	1.5202 ± 3.216 40% n=70	20.2737 ± 17.5 52.1% n=70	-300 ± 1098 10000% n=70	343.068 ± 2621.37 208.8% n=70
9.	0.686649 36.8% n1=4 n2=4	15.9792 19.8%	44.4172 1127%	28.2766 74.6%
10.	0.799535 26.4% n1=3 n2=5	14.6099 9.57%	57.3872 1485.3%	26.3132 76.3%
11.	As above n1=3 n2=3	15.2339 14.25%	26.0355 619.2%	38.8382 65.0%
12.	0.697517 35.8% n1=4 n2=4	16.5284 24.0%	40.3764 1015.4%	28.3684 74.5%
13.	1.03834 4.39% n1=3 n2=5	13.6633 2.47%	59.1974 1535.3%	26.3796 76.3%
14.	As above n1=3 n2=3	14.2206 6.65%	23.8578 559.1%	41.1981 62.9%

Pegg (1969) calculated transcortin capacity graphically by drawing asymtotic lines through the extreme ends of the curve; a perpendicular dropped from the intersection of these two lines to the abscissa yielded the value of P1. K1 was determined from the slope of the initial part of the curve in the same manner as described for the limit method. P1 calculated by Pegg's method for the test data was 14.57 mcg% (9.3% error) (Figure 6) a somewhat larger error than by the limit method (Table 2 part 14). No estimate of albumin binding parameters could be made by Pegg's method.

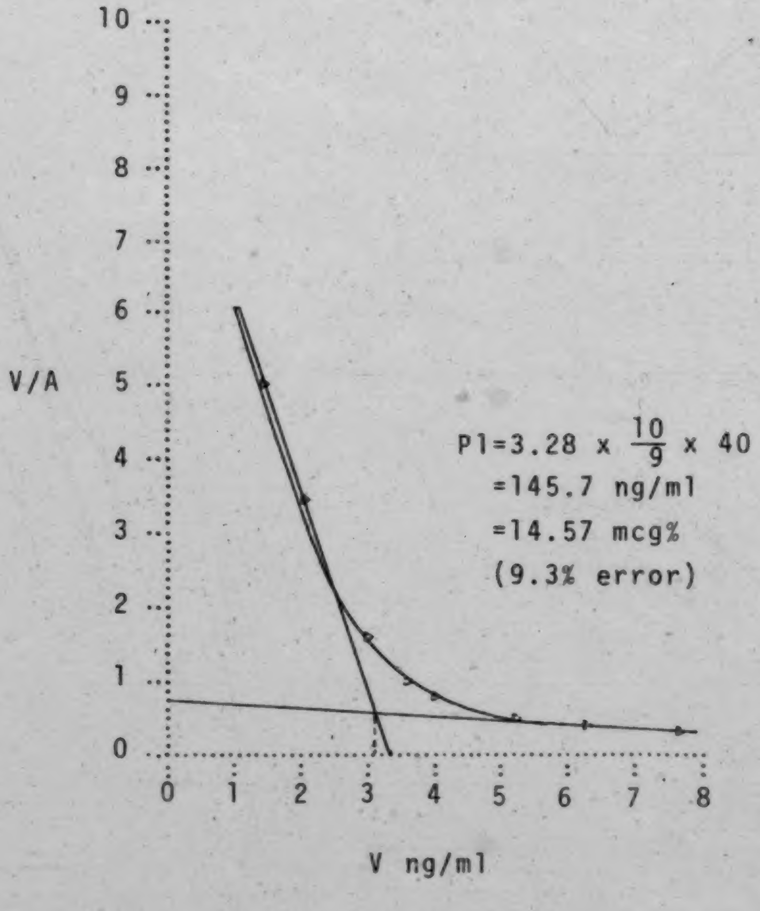


FIGURE 6 Simulated Protein Binding Data; Estimation of [illegible]1 by Method of Pegg (1969).

Iterative approaches to finding the best fit line for binding data have been described in the literature (Fletcher, 1968; Keller, 1966); but the mathematical and the limit method of resolving binding data offer the advantage of simpler computation which can be handled with a small laboratory computer (8K memory). For large amounts of data automated techniques of data handling are necessary; graphical techniques are too tedious although they offer the advantage of simplicity. The two methods described here give reasonably good estimates of transcortin binding parameters; however albumin binding parameters are not estimated accurately. This may however be a function of the range of the data used.

(ii) PRACTICAL CONSIDERATIONS

Several approaches have been used to measure bound and unbound cortisol. Use of radioactively labelled cortisol greatly aids in the detection of small amounts of cortisol. Equilibrium dialysis was probably the first approach used (Daughday, 1956). Here the protein solution is contained within a semipermeable membrane which allows passage of solvent and small molecular size solutes but not protein and protein bound solute. A known concentration of cortisol is added to the system, either inside or outside the membrane, and allowed to equilibrate. Because of the binding of cortisol to protein the concentration inside the membrane is higher than outside, the difference accounting for the amount of cortisol bound. At equilibrium the unbound concentration of cortisol is equal on both sides of the membrane. Bound plus unbound, unbound and total cortisol concentrations are measured, bound concentrations being obtained by subtraction. The solutions on each side of the membrane must be isotonic to prevent osmotic flow. This is most easily achieved by dilution of the sample with isotonic buffer.

Another commonly used method is ultrafiltration (Sandberg, 1957). Here a protein solution is filtered through a semi-permeable membrane or membrane filter of proper pore size and a protein free solution of unbound cortisol is collected. Force for filtration is usually provided by centrifugation or by vacuum. If small volumes of protein solution are used the equilibrium of bound and unbound will be upset during filtration. Usually unbound is measured and bound is obtained by subtraction from total cortisol concentration.

Gel filtration can also be used to physically separate and measure bound and unbound cortisol (DeMoor, 1962). An equilibrated solution of protein and cortisol is passed through a column of gel particles. The free cortisol enters the beads of gel and its passage through the column is slowed, while the bound cortisol is excluded from the gel and it is eluted from the column sooner. Separation on the column must be sufficiently rapid to avoid on-column dissociation of the cortisol protein complex. The half time for dissociation of cortisol is 25 minutes at 4°C, 1 minute at 22°, and 10 seconds at 37° (Dixon, 1968) therefore gel filtration must be done at a lowered temperature. Recently Paterson (1973) found dissociation half times for cortisol/transcortin to be 20 minutes at 2°C, 1 minute at 22°, and 0.24 minutes at 37°, and Rosner (1973) found the dissociation half times to be 5 minutes at 5°C, 2.2 minutes at 10°, 0.8 minutes at 18°, 0.49 minutes at 22°, and 0.087 minutes at 37°.

Several less commonly used methods for determination of bound and unbound cortisol have been described. Westphal (1955) used ultracentrifugation to study cortisol albumin binding. The upper portion of the tube contained free cortisol from which unbound cortisol could be accurately estimated. A gradient of protein in the lower portion of the ultracentrifuge tube rendered measurement of bound cortisol inaccurate, therefore bound was most accurately obtained by subtraction of unbound from total cortisol concentration. Westphal also used electrophoresis in an electrophoretic cell to separate Albumin bound and free cortisol, the bound cortisol migrating in the electrical field while the free cortisol remained stationary. Paper electrophoresis was not successful with cortisol because it tends to bind strongly to the paper upsetting the equilibrium of bound and free steroid.

Isoelectric focusing, an electrophoretic technique which separates proteins according to their isoelectric point, has been used to study the mechanism of cortisol/transcortin interaction although not to give quantitative results. The technique can detect charge alteration due to binding of non-ionic ligands provided the ligand is bound to the protein at its isoelectric point. Native transcortin does not bind cortisol at its isoelectric point, but sialic acid free transcortin does. From this technique it was shown that association of cortisol is accompanied by liberation of protons and dissociation is accompanied by uptake of protons (Baelen, 1972).

Leybold (1967) used enzyme degradation of cortisol to measure cortisol binding capacity of plasma. The plasma was incubated with an excess of cortisol, then NADPH and rat liver microsomes were added to the solution. Microsomal delta^4-3-keto steroid hydrogenases reduced only the nonprotein bound cortisol to tetrahydro-5-alpha-cortisol which is nonfluorescent. The cortisol and metabolites are extracted and measured fluorimetrically. Cortisol measured indicates binding capacity. The flaw in this method is that during the half hour incubation with the enzymes at 37°C considerable dissociation of cortisol/transcortin would occur.

Partition equilibrium between two aqueous phases, formed by mixing 10% dextran of molecular weight 400,000 and 7% polyethylene glycol of molecular weight 6000, in phosphate buffer, is similar in principle to equilibrium dialysis provided the binding protein is quantitatively partitioned into one phase (Shanbhag, 1973). The partition constant of the steroids between the two phases determines the efficiency of free steroid removal from the phase containing the protein. Equilibration can be achieved more quickly than with equilibrium dialysis because better mixing is possible. The approach has been applied to the study of testosterone binding to sex hormone binding globulin and albumin.

Adsorbents (eg. Fuller's earth, florisil, charcoal) which remove free cortisol from solution can be used to separate bound and free steroid (Heyns, 1967). Bound and total cortisol can be measured , unbound being obtained by subtraction. Adsorbents compete with the binding protein for steroid if left in contact with the steroid protein solution sufficiently long. If the time of exposure to adsorbent is short in terms of the cortisol transcortin dissociation rate, the adsorbent will remove only free steroid. This means the technique must be used at lowered temperatures. Adsorbents are less than 100% efficient in removing free steroid, so a correction for this needs to be made otherwise bound will be overestimated. The amount of steroid adsorbed depends on unbound concentration of steroid.

Let B' = apparent steroid concentration, measured

U' = apparent unbound steroid concentration, = T - B'

T = total steroid concentration, measured

U = true unbound steroid concentration

B = true bound steroid concentration

E = fractional efficiency of adsorbent for removal of U, determined by comparison to a method with known efficiency for removal of unbound steroid.

Therefore

$(T - B') = U' = U \times E$

rearranging $U = (T - B')/E$ (14)

$B = T - U$ (15)

The amount of adsorbent also effects the efficiency of free steroid removal. An amount should be chosen such that most of the free steroid is removed but 'stripping' of steroid from protein does not occur. The method is usually called competitive adsorption. Heyns (1967) and DeMoor (1968) have used competitive adsorption to determine cortisol binding affinity in diluted plasma the capacity of which was determined independently. Pegg (1969) used competitive adsorption to determine both transcortin capacity and affinity from a binding curve. Pegg's procedure involved equilibration of the plasma with tritiated cortisol at 45°C, cooling for 10 minutes by immersion in a bath at 6°C, then addition of adsorbent, mixing and centrifugation all at 6°C. Pegg interpreted the parameters as being determined at 6°C which appears to be incorrect. The initial equilibration was done at 45°C for 5 minutes. Association rate for cortisol with transcortin is very rapid; dissociation rate is the slower and is the limiting factor in achievement of equilibrium between free and bound cortisol. At 45°C cortisol and transcortin dissociate quickly (dissociation half time < 10 seconds, Dixon, 1968, first order dissociation rate = 0.0693 seconds^{-1} = 4.2 minutes^{-1}) in five minutes at 45°C the cortisol and transcortin will have equilibrated (1 x $e^{-5\cdot4}$ =0 cortisol remains associated to the initial transcortin binding site). The solution is then placed in a bath at 6°C for 10 minutes. During this time the solution cools (but not instantaneously) all the while cortisol and transcortin continue to associate and dissociate. Dissociation becomes slower as the temperature drops. It can be shown that in 10 minutes the cortisol transcortin will not have reached equilibrium for that temperature. At 6°C dissociation half time is 16.7 minutes (first order dissociation rate =0.042 minutes^{-1}). In ten minutes at 6°C ($e^{-10\times0.042} = 0.66$) 66% of the cortisol will not have dissociated from its original binding site on transcortin. Obviously cortisol and transcortin do not reflect conditions for equilibration at 6°C. Because the cooling from 45°C to 6°C is not instanteous the temperature condition of the system is ambiguous. Actual dissociation will be greater than estimated by the above calculation because dissociation rate is greater than 0.042 minutes^{-1} during the cooling to 6°C. Now adsorbent is added to remove free steroid before equilibrium is reached. The temperature at which the binding parameters are determined is ambiguous because of failure to reach equilibrium and because of the slow rate of cooling of the solution. DeMoor and Heyns avoided ambiguity by doing all work at room temperature. At this temperature however there is considerable dissociation of the cortisol transcortin during exposure to the adsorbent. Doing all work at 8°C avoids the problem of ambiguity and reduces the amount of dissociation of protein cortisol complex during exposure to the adsorbent. Equilibration times of at least 3 hours are required for the protein and steroid to come to equilibrium. At 8°C the dissociation half time of cortisol transcortin is 11.7 minutes (dissociation rate = 0.059 minutes^{-1}, 0.0001 =

$e^{T\times -0\cdot 059}$, T = 156 minutes = 2.6 hours where T = time in minutes for all but 0.01% of the cortisol transcortin to dissociate). During an exposure to adsorbent for two minutes some dissociation will take place ($e^{-2\times 0\cdot 059}$ = 0.89, that is 89% of cortisol transcortin is associated with its original binding site).

Several factors are important in the determination of cortisol protein binding parameters. Percentage binding of cortisol to plasma varies inversely with temperature between 4°C and 45°C (DeMoor, 1962). This is because transcortin binding affinity is higher at lower temperatures (Daughday, 1961; Burton, 1972; Westphal, 1967; Goldie, 1968) albumin binding affinity for cortisol is relatively unaffected by temperature (Burton, 1972). Transcortin binding capacity is not affected by these kind of changes in temperature (Westphal, 1967; Goldie, 1968). Murphy (1963) and Seal (1963) presented data showing that binding capacity was greater at 9 or 4°C than at 37°C. Seal (1963) determined binding capacity by overloading plasma with cortisol, then removing the unbound cortisol by gel filtration, and finally determining the cortisol remaining in plasma. With gel filtration on-column dissociation of cortisol and transcortin would occur at 37°C giving a falsely low binding capacity. Murphy (1963) obtained binding capacity by extrapolation of a plot Of log total cortisol versus percent unbound cortisol to zero unbound. The total cortisol at zero unbound was taken to be equal to binding capacity. The relationship log total versus percent unbound is not linear at high values of percent unbound (Figure 7, obtained with simulated data). At 9°C only short extrapolation is needed and only a small error occurs. Murphy admits that at 37°C extrapolation over long distances was necessary because binding was lower. This extrapolation would lead to large errors in estimates of binding capacity (Figure 7) thus these results suggesting that binding capacity increased with decreasing temperature are incorrect. Transcortin binding capacity is not affected by moderate changes in temperature. Temperatures above 60°C will irreversibly inactivate transcortin binding without affecting albumin binding (Daughday, 1961). This provides a means of differentiating albumin and transcortin contributions to total binding of cortisol by plasma.

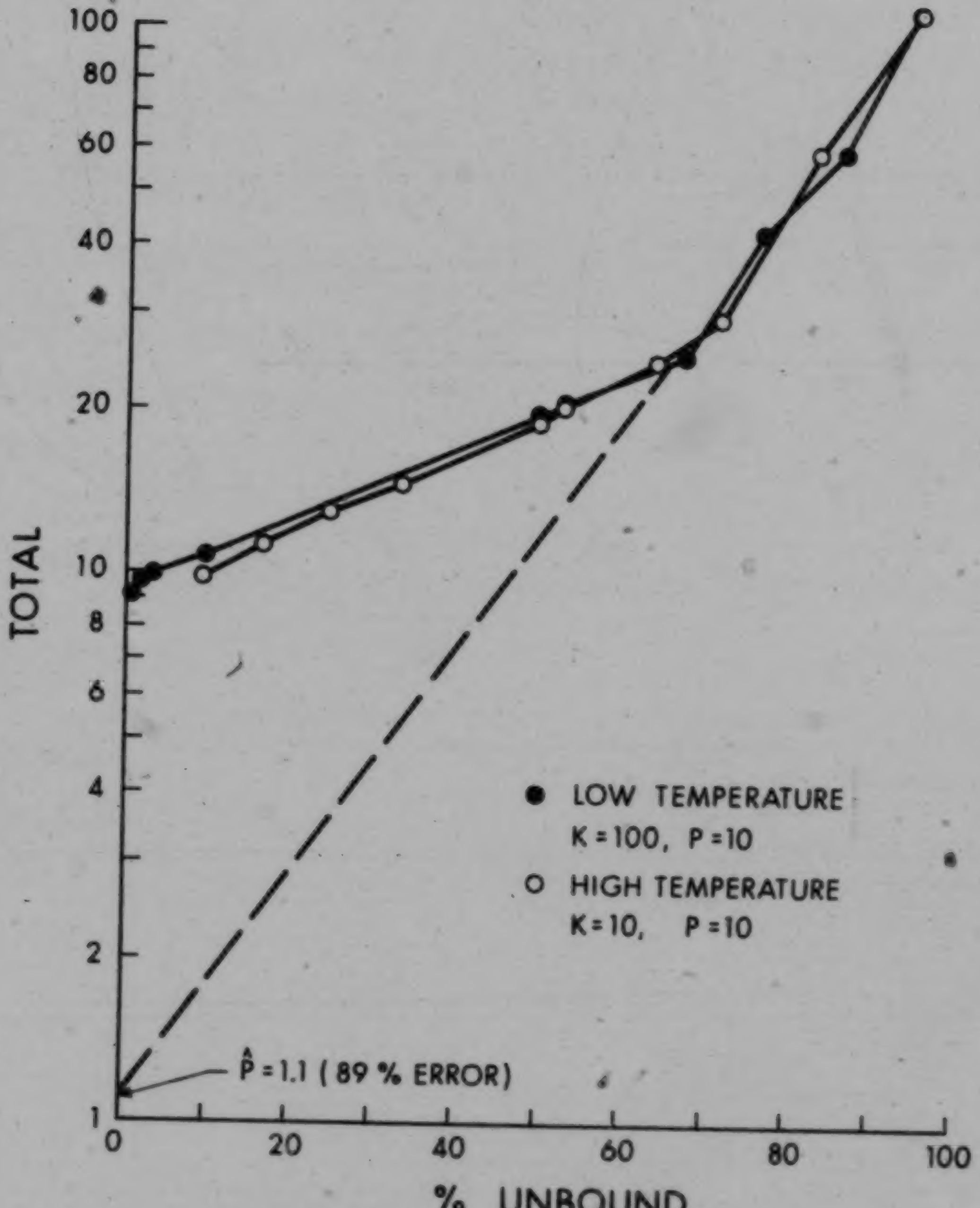


FIGURE 7 Simulated Single Component Binding Data; Calculation of P by Method of Murphy (1963).

Transcortin is unstable at pH's less than 5 (Daughday, 1961). Binding by transcortin is stable throughout pH ranges attainable in vivo (Daughday, 1961). At temperatures from 0° to 40°C, pH changes from 7 to 8 cause only a 1% decrease in unbound cortisol in plasma (Chen, 1961); therefore the statement of DeMoor (1968) that minor changes in pH affect binding seems to be unfounded.

Dilution of plasma is often required in determination of its binding activity. This does not affect transcortin binding capacity (Murphy, 1963). It is often a useful technique because dilution hastens equilibration (Daughday, 1961), makes differences in binding more pronounced (Slaunwhite, 1959) and allows determination on small volumes of plasma. Correction of binding parameters for dilution is simply a mathematical exercise. Dilution of plasma results in a lowering of percent binding for a given cortisol concentration in plasma (eg undiluted plasma 85% cortisol is bound, 1 in 20 dilution of the same plasma 35% cortisol is bound, endogenous cortisol concentration 20.5 mcg%, Beisel, 1964b).

When binding parameters for multiple samples are to be determined, storage of plasma prior to binding assay needs to be considered. Freezing at -15°C or refrigeration at 4°C does not affect the cortisol binding index of plasma. The cortisol binding index is the product of cortisol binding affinity and cortisol binding capacity (DeMoor, 1968).

Table 3 is a list of cortisol binding parameters reported in the literature. Except in the cases discussed above it can be seen that transcortin binding affinity increases with decreasing temperature but transcortin binding capacity is unaffected by changes in temperature. Various methods of determining binding parameters give similar results.

TABLE 3 CORTISOL BINDING PARAMETERS OF HUMAN PLASMA

TRANSCORTIN BINDING PARAMETERS

P_1' mcg%	K1 l/m $\times 10^8$	Sample	Temperature Solvent	Method	Reference
n=1	0.3	purified transcortin	37°C		Burton, 1972
3.2		serum over-loaded with cortisol	37°C	gel filtration	Seal, 1963
22.8	1		37°C, pH 7.4 phosphate buffer	ultra-filtration equilibrium dialysis	Goldie, 1969
20-30	0.36-0.72		37°C		Mills, 1962
77-90	0.11-0.19	estrogen			
15.4	0.47	plasma diluted 1 to 2	37°C saline	ultra-centrifugation	Beisel, 1964b
21.3± 2.2	0.42± 0.1	diluted plasma	37°C	ultra-filtration	Murray, 1967
26.1± 4.3	0.74± 0.21	undiluted plasma	37°C	ultra-filtration	Keane, 1969
53± 15.6	0.58± 0.16	pregnancy			

27.8	0.55	cirrhosis			
25.9 ± 3.8		plasma overloaded with cortisol	25°C,0.15M phosphate buffer pH 7.35	gel filtration	DeMoor, 1962
15.7± 1.3 35.3-36.7		as above estrogen	as above	as above	Smith, 1969
24.2± 2.5	0.74	plasma cleared of cortisol and diluted 1 to 4	25°C,0.02M phosphate buffer pH 7.3 in 7% bovine serum albumin	competitive adsorption and gel filtration	DeMoor, 1968
n=1	0.52	purified transcortin	23°C		Rosner, 1973
30.9± 4.5 31.8± 5.5 24.3± 5.1 67.5± 9.9		females males males cirrohotic males estrogen plasma diluted 1 to 10	9°C,0.9% NaCl	dialysis	Murphy, 1963
	1.7	purified transcortin	6°C	equilibrium dialysis	Schneider, 1971
23± 2.8	4.2± 1.2	plasma	6°C	competitive	Pegg, 1969

		diluted 1 to 62.5	water	adsorption	
46.7± 7.1	3-5.3	pregnancy			
45, 48	6.7	oral contraceptives			
	5	purified transcortin	4°C		Seal, 1966
	5.2	as above	4°C		Muldoon, 1967
n=1	5	as above	4°C		Burton, 1972
	6	serum	4°C		
28	6	sera cleared of endogenous cortisol and diluted 1 to 10	4°C,0.05M phosphate buffer pH 7.4	multiple equilibrium dialysis	Westphal, 1967
22		serum over-loaded with cortisol	4°C	gel filtration	Seal, 1963
	7		4°C		Daughday, 1960
25.3	6	plasma cleared of cortisol	4°C	equilibrium dialysis	Westphal, 1966
51	6	pregnancy			

	6	purified transcortin	2°C	equilibrium dialysis	Seal, 1962

ALBUMIN BINDING PARAMETERS

P2 mcg%	K2 1/m	Sample	Temperature	Method	Reference
	10^{-3} to 10^{-5} times K1	serum cleared of endogenous cortisol and diluted 1 to 10	37°C, 0.05M phosphate buffer pH 7.4	multiple equilibrium dialysis	Westphal, 1967
	6×10^4	serum	37°C		Burton, 1972
	n X K2 = 5×10^3		5°C, pH 7		Sandberg, 1957
n1=1 n2=20	$k=3.6 \times 10^3$ $k=1.2 \times 10^2$ $n1k+n2k=6 \times 10^3$	0.7% human serum albumin	5°C, pH 7		Slaunwhite, 1963
	10^4				Lipsett, 1972
900-1800 mg for total body	$2-5 \times 10^3$				DeMoor, 1964

E. DISTRIBUTION OF GOLD IN THE BODY

The distribution of gold compounds has been studied in several species. The metabolism or biotransformation of gold compounds has not been studied to any great extent. This is because most of the available methodology detects gold but not the chemical compound. Several authors have misnamed distribution studies with gold as metabolism studies. Kinetics of gold distribution have been studied to a limited extent but these studies have not attempted to explain gold kinetics in terms of formalized pharmacokinetic models. Gold exhibits complex pharmacokinetics which cannot be explained by simple first order rate constants and one compartment models although over specified periods of time first order kinetics are approximated. Water soluble gold compounds, which are the compounds used in RA therapy, are distributed differently from colloidal gold. The distribution of colloidal gold depends more on its physical size while that of non-colloidal gold compounds depends on their chemical properties. This review is confined chiefly to water soluble non-colloidal gold compounds. Most water soluble gold compounds are well absorbed intramuscularly (im) but are poorly abosrbed orally. The gold concentrates in the liver, kidney, spleen and adrenals, tissues with high content of reticulo-endothelial cells (RES). Gold appears in blood quickly after im injection but levels decline rapidly due to tissue distribution rather than rapid urinary or fecal excretion. Gold can be detected in urine and blood long after gold therapy is stopped, presumably due to a slow return of gold from tissue stores. The slow development of a therapeutic response to gold suggests that the site of action of gold may be some deep tissue site which only slowly accumulates therapeutic concentrations of gold.

Gold, as a water soluble complex, is rapidly absorbed from im injection sites. Peak gold levels in plasma are reached in 2 to 4 hours for gold sodium thiomalate, aurothioglucose, and gold sodium thiosulfate administered im to RA patients. (Freyberg, 1942; Lorber, 1970; Oka, 1973; Pole,1971; Rubenstein, 1973). Absorption is not complete, since tissue distribution studies in animals usually reveal considerable amounts of gold remaining at the injection site (Block, 1941) while scanning studies in humans given ^{198}Au also indicate considerable activity at the injection site (Lawrence,1961; Oka,1973). Ninety days after completion of a series of 14 x 1mg Au/d (all doses are given in terms of elemental gold content) im injections in rats the amount of gold absorbed varied from 58% for aurothioglucose, to 69% for gold sodium thiomalate, to 70% for gold calcium thiomalate, to 82% for gold sodium thiosulfate, to a high of 90% for sodium succinimidoaurate (Block, 1944). Gold sodium thiomalate is only minimally absorbed when given orally. A

20 mg/kg/d dose of gold sodium thiomalate given to rats produced a serum level on day 17 of 0.7 mcg/ml compared to a serum level of 19.4 mcg/ml when the same dose was given im (Walz, 1972). Chloro(triethyl-phosphine)gold, a compound which is more lipid soluble than other gold compounds used therapeutically, gave appreciable serum levels when given orally to rats. A dose of 20 mg/kg/d gave a serum level of 4.6 mcg/ml on day 17; the same dose of gold sodium thiomalate im gave a serum level of 10.6 mcg/ml on day 17 (Walz, 1972; Sutton, 1972). Gold sodium thiomalate was absorbed systemically when given intra-articularly (ia) giving a peak blood level at 3 hours. This level was lower than that following a similar dose by im injection (Oka,1973).

Gold rapidly appears in the blood after administration; however it is quickly distributed to tissues so that plasma levels are similar 4 hours after intravenous or intramuscular administration of gold sodium thiosulfate despite incomplete absorption in the latter case (Freyberg, 1942). The decline in the level of plasma gold is roughly exponential during the first ten to 42 days (Gerber, 1972a; Rubenstein, 1973) so plasma half life can be estimated. It varies from 3.97 to 9 days in rheumatoid arthritis patients (Gerber, 1972a, 1972c; Rubenstein, 1973; Babb,1967). After this time gold levels decline much more slowly (Mascarenhas, 1972). Plasma gold levels depend on dose, frequency of dosing and time of sampling. In addition plasma gold levels have been shown to be negatively correlated with the weight of the patient (Jessop, 1973a). A few examples of plasma gold levels achieved with various dosage regimes will be given in order to illustrate plasma levels in therapy with gold. Plasma levels within 2 to 4 hours of dosing have been reported as 2.5 mcg/ml at 12.5 mg (first dose), 4 mcg/ml at 20-25 mg (first dose), 3.8 mcg/ml at 12.5 mg (after previous doses), 5.4-9.3 mcg/ml at 25 mg (after several previous doses) of gold sodium thiomalate im (Pole, 1971; Lorber, 1970; Rubenstein, 1973). Aurothioglucose gave lower plasma levels at this time: 1 mcg/ml at 12.5 mg (first dose), 2 mcg/ml at 25 mg (first dose), and 4 mcg/ml at 25 mg (after several previous doses) (Rubenstein, 1973). One day after injection plasma gold levels were declining: 2 mcg/ml after 20 mg (first dose), and 4.5-6.2 mcg/ml after 25 mg (after several previous doses) of gold sodium thiomalate (Krusius, 1970;Lorber, 1970; Pole, 1971). The decline continued so that after one week plasma levels were 1.8 mcg/ml at 20 mg (first dose, 1.8-4.2 mcg/ml at 25 mg, 5-6.6 mcg/ml at 38 mg (latter two doses were both given after several previous doses) (Pole, 1971; Gerber, 1972b; Krusius, 1970; Lorber, 1970). Five weeks after the dose was given plasma levels were 1 mcg/ml for 20 mg (first dose) and 1.7-2.7 mcg/ml for 25 mg (after several previous doses) of gold sodium thiomalate (Gerber, 1972b; Pole, 1971). Gold can be detected in plasma for very long periods of time after therapy with gold has ceased, for example Plantin (1961) reported plasma gold levels of about 0.1 mcg/ml several years after gold therapy.

Gold circulates in the plasma almost completely (95-99%) protein bound (Sorensen, 1970; Sliwinski, 1968; Mascarenhas, 1972). It is bound chiefly (75%) to albumin (McQueen, 1969; Sorensen, 1970; Bellion, 1957; Eberl, 1970; Sliwinski, 1968), but also to alpha and beta globulins (Bellion, 1957; Eberl, 1970; Coke, 1963; Sliwinski, 1968) and at higher plasma gold levels (3-7 mcg/ml) it was bound to immunoglobulins and beta-1-c-complement (Lorber, 1972a). Binding to fibrinogen does not occur since plasma and serum levels of gold are very similar (McQueen, 1969; Smith, 1973; Balzacs,1972). Gold was not found to bind to isolated fibrinogen (Sliwinski, 1968). More specific studies show gold is bound to alpha-1-globulins, alpha-2-globulins (perhaps ceruloplasmin), beta-1-globulins (perhaps transferrin) and to beta-lipoproteins which contain some gamma globulin (Clemmensen, 1958; Coke,1963; Eberl, 1970). It is of interest to note that transcortin is an alpha-1-globulin.

In most cases gold is not found in red blood cells

(Block, 1941; Balzacs, 1972; Freyberg, 1942; Harth, 1973) nor is it found in white blood cells (Sliwinski, 1968). Lawrence (1961) reported 0.4 mcg/ml of gold in red blood cells one day after a 5 mg dose of gold as thiomalate salt, compared to plasma levels of 1.5 mcg/ml. Smith (1973) found gold in the red blood cells of 10 of 16 patients studied. Serum levels of gold were similar in all patients regardless of presence of gold in the red blood cells. Gold did not appear in the red blood cells until after some delay from the start of gold therapy. They suggested that gold was incorporated into the red blood cells in the bone marrow when the cells were formed. Autoradiography of blood smears from patients given tracer ^{195}Au gold sodium thiomalate showed gold incorporation into only a portion of the red blood cells (Oka, 1973). This supports the suggestion that gold is incorporated into the red blood cells in the bone marrow, since only newly formed cells should show any gold while older cells in circulation prior to the ^{198}Au dose would not contain any activity.

Tissues distribution studies in many species (guinea pig, dog, rat, mouse, rabbit, man) with several forms of soluble gold compounds (gold cyanides, gold chloride, gold sodium thiosufate, aurothioglucose, gold sodium thiomalate, gold calcium thiomalate) reveal that the spleen, kidney, adrenal, bone marrow, lymph nodes and liver accumulate the highest concentration of gold, concentrations much higher than found in plasma (DeWitt, 1918; Elftman, 1946; McCluskey, 1925; Bertrand, 1948; McQueen, 1969; Swartz, 1960; Tobias, 1949; Vacik, 1961; Block, 1941, 1942; Jeffrey, 1958; Erametsa, 1970; Gallinal, 1927; Gottlieb, 1972a; Oka, 1973; Sairanen, 1973). The organ from this group with the highest concentration varies from one report to another but does not seem to be related to chemical form of gold, species of animal, or temporal factors. Tissue levels for this group of organs are approximately 40 mcg/g (range 1-100 mcg/g) calculated for total gold dose of 10 mg/kg. All these organs are part of the reticulo-endothelial system (Bell, 1965; Brun, 1964; Dougherty, 1961). RES cells of the adrenal cortex are able to 17-hydroxylate progesterone more than other cells in the adrenal (Dougherty, 1961) so it is possible that accumulation of gold in the adrenal could result in stimulation of RES and also stimulation of cortisol synthesis. This suggests that soluble gold compounds may be reduced in the body to metallic gold particles which are picked up the the phagocytic RES cells. Microdistribution of gold in the kidney of rats, guinea pigs and humans showed concentrations of gold particles revealed by histochemical methods or electron microscopy were greatest in the proximal convoluted tubules (Gilg, 1952; Silverberg, 1970; Elftman, 1946; Strunk, 1968; Brun, 1964). In the proximal tubule, gold was present in mitochondria.

Concentration of gold within the organelles caused destruction of the mitochondria (Stuve, 1970). Gold was also found in the glomerulus but in lower concentrations (Strunk, 1968, 1970; Brun, 1964). Here it is usually contained within lysosomes. Gold was occasionally found in the loop of Henle and in the distal tubules at still lower concentrations and was not found in the collecting ducts in renal biopsies from RA patients at various stages of gold therapy (Brun, 1964). Deposits of gold appeared in the proximal tubules within hours to months after starting gold sodium thiosulfate (Brun, 1964; Ganote, 1966) and disappeared 1 to 4 years after gold was stopped (Brun, 1964). At this time gold was found in epithelium of the distal tubules. Gold was also found in interstitial cells of the kidney; these cells are RES cells. These microdistribution studies suggest gold may be excreted by secretion at the proximal convoluted tubule. Since gold is highly protein bound, filtration at the glomerulus would be reduced. In nephrosis, abundant deposits of gold are found in glomerular capillary walls and small deposits in capillary basement membranes (Strunk, 1968,1970). In the liver, gold was found chiefly in the Kupffer cells, which are RES cells, and only occassionally in hepatic cells (Gilg, 1952; Elftman, 1946); thus gold probably has no effect on drug metabolism, but may affect steroid metabolism since RES cells of the liver and other tissues can metabolize steroids by A ring reduction (Dougherty, 1961).

Distribution of gold to certain other tissues and fluids of the body is of particular interest because of the special significance of these tissues in rheumatoid arthritis, despite the fact that the concentration of gold is often lower than in the organs mentioned above. One such group of tissues and fluids is in the joints. Synovial fluid concentrations of gold parallel plasma gold levels (Clausen, 1962b); synovial fluid concentrations usually amounted to 50-60% of plasma concentrations (Gottlieb, 1972a; Lawrence, 1961; Lorber, 1970; Sliwinski, 1968; Gerber, 1972a; Freyberg, 1941) although occasional cases had higher synovial fluid concentrations (Hartung, 1940b). The half-life of gold in serum and in synovial fluid was found to be the same (Gerber, 1972a). Inflamed and painful joints accumulate greater concentrations of gold than do non-inflamed joints (Bertrand, 1948; Lawrence, 1961; Oka, 1973; Sairanen, 1973). This effect is not specific for articular tissue since a similar effect can be demonstrated in non-inflamed and inflamed extra-articular tissue (Bertrand, 1948). The concentration of gold in the synovium was lower than that of synovial fluid (Bertrand, 1948; Lawrence, 1961). Lawrence had reported high levels of gold in fibrin clots from joints and in articular cartilage but he, himself, pointed out the values were suspect because of small sample size relative to the other samples used in the study. Microdistribution studies, by light and electron microscopy, and by autoradiography, of synovia from RA patients treated with gold, revealed gold granules in the cytoplasm of synovial lining cells and in macrophages around lymph follicles and blood vessels in the subsynovial layer (Inoune, 1968; Oka, 1973). The gold was found inside lysosomes of macrophages. Paltemaa (1968b) using rat liver suspension showed that gold was taken into these cells in amounts proportional to the amount of damage to the lysosomes. He used vitamin A in large doses to damage the lysosomes. He suggested that gold was more easily taken up into damaged lysosomes such as may be found in RA.

Gold distribution to tissues involved in adverse reactions to gold is also of interest. The most frequently involved tissues in adverse reactions to gold are the kidney, which has been discussed above, and the skin. Deposition of gold in the cornea of the eye has been reported (Hashimoto, 1972) especially at doses greater than 750 mg. This deposition does not appear to be a serious effect since visual disturbances did not occur. Skin gold levels in animals treated with gold sodium thiosulfate in doses of 1 - 5 mg/kg were 0.1 to 4.7 mcg/g and were related to dose (Bertrand, 1948; Tobias, 1949). In humans skin gold levels of 0.019 mcg/g for a dose of 2.5 mg of gold as gold sodium thiosulfate (Bertrand, 1948), 0.5 mcg/g for a dose of 5 mg of gold as sodium thiomalate (Lawrence, 1961), 0 - 40 mcg/g for weekly doses of 10 to 100 mg of various gold salts (Freyberg, 1942) and 38 mcg/g in an area of skin not affected by dermatitis for a total gold dose of 2.5 g as thioglucose (Gottlieb, 1972a) have been reported. Freyberg (1942) could find no association between skin gold levels and dermatitis, while in a single case Gottlieb (1972a) found higher gold levels in an area of skin affected by dermatitis (79 mcg/g) compared to 38 mcg/g reported for skin with no dermatitis. In a series of 22 patients, 4 with gold dermatitis, Gottlieb (1974a) found no difference in skin gold level in patients with and without dermatitis nor in normal and abnormal skin from patients with dermatitis. Skin gold levels were about 4 mcg/g at total gold doses of about 300 mg. Bogg (1958) found 20 mcg/g in skin taken from the shoulder and the thigh of a patient who exhibited generalized dermatitis after about 4 g of gold. Skin biopsies were taken three years after gold therapy had been stopped although dermatitis persisted. In three other cases gold concentrations were 85, 30 and 50 mcg/g in areas of abnormal skin and 40, 10 and 15 mcg/g, respectively in normal skin. Microscopic examination of skin from patients with chrysiasis, which is a permanent pigmentation (varying from blue to gray) of the skin due to parenteral gold administration and subsequent exposure to ultraviolet light, shows granules of gold in the dermis (Zimmerli, 1929), in the basal layer of the epidermis (Montgomery, 1967; Edstrom, 1959), in subcutaneous tissue (Edstrom, 1959), in dermal histiocytes (Cox, 1973), in melanophages and in blood vessel walls (Montgomery, 1967). There are no reports of quantitation of gold in the skin in cases of chrysiasis.

Levels of gold in hair and nails are of interest because these tissues are easily accessible for sampling. Gottlieb (1972a) detected 5 mcg/g in the fingernails and >2 mcg/g in the hair of a patient who had received 2.5 g of gold as aurothioglucose. In a group of four patients treated for one year with gold to a total dose of 600 mg gold average hair gold levels were 0.58 ± 0.40 mcg/g and average nail gold levels were 0.74 ± 0.66 mcg/g (Gottlieb, 1974b).

In longterm studies of the distribution of gold, levels of gold become quite small so it is necessary to know the normal level of gold which is present in an individual who has never been treated with gold. Neutron activation analysis provides sufficient sensitivity for these measurements. Blood gold levels in persons never treated with gold have been reported as 5.5×10^{-11} g/g up to 10^{-9} g/g (Brune, 1966; Botzvadze, 1969; Plantin, 1961). The saliva gold level was reported as 6×10^{-10} g/ml

(Kanabrocki, 1965). Fingernails contain 6 x 10^{-9} to 5 x 10^{-7} g/g (Petushkov, 1969; Kanabrocki, 1968). Skin gold levels of 2.2 x 10^{-9} g/g of air dried skin were reported (Feuerstein, 1967). Liver gold levels were reported as 1.3 to 70 x 10^{-11} g/g with a median of 5.7 x 10^{-11} g/g and a mean of 1.14 x 10^{-10} g/g (Parr, 1963). In this study gold levels in liver did not increase with age. Tipton (1963) using a spectrographic method and using samples from 250 individuals who died accidently, found median trace levels of gold in all tissues examined to be <10 mcg/g of ashed tissue. Most body tissues were examined but in most cases the gold level was below the sensitivity of the analytical method.

Information on the metabolism of gold compounds is scarce and what is available is often based on assumptions for which there is little supportive evidence. Metabolism of gold compounds involves the biotransformation of gold compounds through the action of enzymes. Transformations which occur non-enzymatically are not really metabolism, but will be included here. Elftman (1946) stated that gold was present in nonmetallic form in rat or guinea pig tissue after intraperitoneal administration of gold chloride. This was based on the fact that gold in tissue can be reduced by stannous chloride to give metallic colloidal gold, which is soluble in aqua regia. In previous work (Zwemer, 1946), gold chloride administration had been shown to decrease plasma ascorbic acid (a reducing agent). They hypothesized that tissue gold may be deposited as metallic gold due to the reducing action of ascorbic acid; however the distribution of gold did not correlate with the distribution of ascorbic acid. Timm (1933) and Korteweg (1928) reported that microscopic sections from animals treated with gold sodium thiosulfate showed deposits of gold sulfide and metallic gold. Querido (1947) thought gold in tissue was present as a gold sulfide not as metallic gold, since sodium sulfide which dissolves gold compounds but not metallic gold, could remove gold from tissue sections. Swartz (1960) using ^{198}Au and ^{35}S labelled gold thioglucose found different spatial and temporal distribution patterns for the two isotopes. He speculated that the longer retention of ^{35}S was due to metabolic breakdown of gold thioglucose to metallic gold which would be lipid insoluble, and to thioglucose which would be lipid soluble. The levels of ^{198}Au of tissues, except liver and spleen, were lower than the levels of ^{35}S because the ^{35}S form of gold thioglucose retained its lipid solubility and could therefore become intracellular where it would be retained longer and at higher concentrations. Kleinsorge (1959) observed that labelled gold compounds were excreted unchanged in the urine. Thus evidence from the literature does not clearly indicate whether gold compounds remain intact in the body or are chemically or metabolically broken down to yield metallic gold.

Excretion of gold compounds takes place predominantly via the urine. Fecal excretion is usually less than urinary excretion. Excretion via the saliva and milk is very small since detectable levels of gold have not been found (Hartung, 1940b; Sorensen, 1970). Recently Blau (1973) demonstrated comparable levels of gold in human milk (8.6-10.0 mcg/ml) as have been reported elsewhere in plasma. Small levels of gold were found in the infants serum (0.7 mcg/ml) and red blood cells (0.4 mcg/ml). According to most studies with gold sodium thiomalate the peak excretion of gold occurs during the first 24 hours after injection (Babb, 1967; Mascarenhas, 1972; Sliwinski, 1966; Lawrence, 1961; Bertrand, 1948). Highest fecal excretion occurs later on during the first week after dosing with gold (Mascarenhas, 1972). Cumulative gold excretion by both urinary and fecal routes amounts to 16 to 39% (expressed as % of current dose) during the first week after dosing (Kapelowitz, 1964; Gottlieb, 1971; Jones, 1971; Mascarenhas, 1972), 55% by the end of the second week (Gottlieb, 1971a; Mascarenhas, 1972) 67% by the end of the third week (Mascarenhas, 1972), and 77% by the end of the fourth week (Mascarenhas, 1972) for gold sodium thiomalate and aurothioglucose in humans. The excretion of gold calcium thiomalate, a water insoluble salt, is less, amounting to only 44 to 48% after six weeks (Jeffrey, 1958). Fecal excretion varies from 12.5 to 64% of total excretion (Gottlieb, 1971a; Kapelowitz, 1964; Mascarenhas, 1972; Hartung, 1940b; Lorber, 1973; Bertrand, 1948; Block 1941, 1944) although occassionally it is equal to or greater than urinary excretion. Less than 50% of gold in feces can be accounted for by biliary excretion (Kapelowitz,1964). In studies combining ^{195}Au gold sodium thiomalate and neutron activation analysis of gold, Gottlieb (1971a) found that 44% of the excretion was due to currently administered gold and 56% of excretion was due to excretion of previously administered gold in a patient on a schedule of 25 mg/wk. In patients who have received no previous gold the time to excrete half of a tracer dose of gold was 25 days, while in patients on regular gold therapy the time to eliminate half the tracer was 44 days (Gerber, 1972c). One hundred twenty days after administration of the ^{195}Au, 25% of the dose was retained in the body in the first group and 44% was retained in the body by the second group. Urinary clearance rates for the first two days after dosing with gold have been calculated as 0.011 to 0.014 ml/min for a dose of 5 mg/kg and 0.017 to 0.020 ml/min for a dose of 1 mg/kg in rabbits (McQueen, 1969). In humans the urinary clearance rate was calculated to be 0.1 to 0.2 ml/min for a total dose of about 1.4 mg/kg (Mascarenhas, 1972). These clearance data indicate gold is excreted very slowly. Thus on a weekly or even a monthly dosage schedule considerable build up of gold in the body would occur unless some other route of excretion of gold exists.

Many attempts have been made to correlate gold plasma levels and gold excretion with response, either therapeutic or toxic. Smith (1958) suggested that urinary hypoexcretion (4 to 10% dose/week) accounted for early toxicity to gold (2 cases of dermatitis), and hyperexcretion (17 to 28.4% dose/week) accounted for the failure to respond to gold (4 cases). Normal excretion in 4 patients with satisfactory response was 14 to 15.4% dose/week. Gottlieb (1972b) in a study of 18 patients newly initiated to gold, failed to find a significantly different urinary excretion rate for patients who responded favourably and those who did not. Patients who experienced toxicity (6 cases of dermatitis, 1 case of proteinuria) failed to show differences in urinary or fecal excretion of gold compared to patients who did not experience toxicity. Patients who responded well to gold had lower fecal excretion of gold than those who responded poorly. Gottlieb postulated that the low fecal excretion might be related to lesser accumulation of gold in the liver leaving more gold available for other sites in the body where it might exert its action. Rothermich (1967), Rubenstein (1973), Silverberg (1970), Mascarenhas (1972), Gerber (1972b), Jessop (1973) and Gottlieb (1974b) failed to find different plasma gold levels in patients who experienced toxicity to gold (18 cases of dermatitis, 5 cases of nephrotic syndrome, 3 cases of hematological alteration, 15 cases of unspecified toxicity) and those who did not experience toxicity (129 cases). No difference in plasma gold levels was found between those patients who responded well (70 cases) and those who did not respond to gold (48 cases) (Rubenstein, 1973; Mascarenhas, 1972; Gerber, 1972b; Jessop, 1973a; Gottlieb, 1974b). Plasma gold levels greater than 3 mcg/ml on day seven after 25 mg/kg were associated with toxicity (9 cases dermatitis, 1 case hematological abnormality, 5 cases unspecified) (Krusius, 1970; Goodwin, 1966; Jessop, 1973a; Poles,1971). These plasma gold levels were significantly higher than those in patients on similar doses who did not develop toxicity. Pole (1971) and Lawrence (1961) reported low plasma gold levels (<1 mcg/ml) associated with adverse reactions; these were thought to be hypersensitivity reactions as opposed to toxic reactions. Krusius (1970) reported significantly higher plasma gold levels in patients responding well to gold compared to those responding poorly for blood samples taken on day 1, 3, 5 and 7 after a 25 mg dose of gold as thiomalate. Gottlieb (1974b) reported lower whole blood gold levels in patients who responded well than in those who responded poorly, despite similar plasma gold levels in the two groups. Lorber (1973) and DeBosset (1973) both demonstrated a higher proportion of successful responses to gold by giving a higher dose of gold (maintained at 25 to 38 mg/wk) which resulted in average plasma gold levels on day seven of 3.2 to 6 mcg/ml without an increase in the incidence or severity of toxicity. These studies showed that doses higher than those commonly used could be safely used if plasma gold levels were monitored to avoid excessive gold levels. DeBosset (1973) found plasma levels greater than 7.5 mcg/ml were poorly tolerated.

When toxicity to gold is severe, increasing the rate of removal of gold from the body is important. Attempts to achieve this have been most successful with chelating agents; other agents have been ineffective. Acidification of the urine by administration of ammonium chloride (Freyberg, 1942) and alkalinization by giving sodium bicarbonate (Hartung, 1940b; Freyberg, 1942) did not alter the urinary excretion of gold nor did it alter plasma gold levels. Administration of vitamin D or calcium also had no effect (Freyberg, 1942). Large doses of vitamin C slightly increased the excretion of gold in the urine but not to a clinically significant extent (Freyberg, 1942). Administration of thiomalic acid to rats increased the urinary excretion to 145% of control excretion and reduced gold concentrations in lung and spleen, apparently by mobilizing gold from these organs and redepositing it in the liver and kidney since the concentration of gold in these organs increased (Rubin, 1967). Freyberg (1942) failed to demonstrate any effect of thiomalic acid as the sodium salt on the plasma gold levels of, or the excretion of gold in RA patients treated with gold. Dimercaprol (BAL) increased urinary excretion of gold to 175 to 185% of control levels in rats and in RA patients (Rubin, 1967; Saphir, 1966). Kidney levels of gold were increased most by BAL compared to several other agents including thiomalic acid and penicillamine, while spleen and lung gold concentrations were decreased in the same animals (Rubin, 1967). Lorber (1972b) demonstrated that N-acetylcysteine increased urinary excretion of gold following 25 mg doses of gold as thiomalate. In RA patients and in rats, penicillamine increased urinary gold excretion to 137 to 230% of control levels with peak effect on the first or second day after the initiation of penicillamine therapy (Rubin, 1967; Eyring, 1963; Kapalowitz, 1964) but with no effect on fecal excretion of gold (Kapalowitz, 1964). In a case report of the treatment of gold dermatitis with penicillamine, urinary excretion increased to a level four times greater than control on the second day of penicillamine adminstration while fecal excretion increased to a level twice the control excretion on the sixth day (Davis, 1969). Plasma levels of gold also increased indicating gold was being mobilized from tissue. On average fecal, urine and plasma gold levels were 150% of control level. Davis cautioned that penicillamine should only be used in persistent reactions, since adverse reactions to penicillamine are common and since many acute reactions to gold clear spontaneously when gold therapy is interrupted. Davidson (1964) and Eyring (1963) reported that penicillamine decreased kidney concentrations of gold while Rubin (1967) found increased gold concentrations in liver and kidney with decreased gold levels in the lung and spleen. Rubin (1967) judged penicillamine to be the best chelating agent to treat gold toxicity because it gave maximum mobilization of gold and although kidney concentrations of gold were increased, the ratio of urine to kidney gold was was highest. Chelation therapy to treat gold toxicity which often effects the kidney would only be beneficial if the therapy enhanced gold excretion from the body, but not if chelation therapy resulted in redistribution of gold with substantial increases in concentrations in the kidney.

F. ANALYSIS OF GOLD IN BIOLOGICAL MATERIALS

Dewitt (1918) carried out the first study of gold distribution in animal tissues using an electro-gravimetric method which consisted of ashing the tissue with acids then electro-deposition of the gold on tared platinum electrodes. The margin of error of the method was described as 0.02 to 0.03 mg. The smallest amount of gold detected was 0.02 mg.

In 1940, Block developed a spectrophotometric method for determination of gold in blood and urine. The samples were acid digested, decolorized with hydrogen peroxide, evaporated and dissolved in aqua regia to convert all gold to auric chloride. A pink colour was developed with 0.1% o-dianisidine in 10% potassium fluoride and 25% hydrochloric acid. The useful range of this method was 0.01 to 40 mg of gold. Beamish (1961) in his review of gold analysis by colorimetric and spectrographic methods noted that Block's method was subject to interference from excess oxidizing agent (aqua regia or nitrosyl chloride). Beamish (1961) in his review included only three other colorimetric methods applied to biological materials. The first used dimethylaminobenzylidenerhodanine in a solution of ethyl alcohol, chloroform and benzene, which developed a pink violet color in the organic layer when mixed with an aqueous solution of gold containing a few drops of nitric acid. The sensitivity was 0.1 mcg/5 ml but the method gave large blanks with tissue samples. A related reagent, diethylaminobenzilidenerhodanine, produced a red violet product with gold. The sensitivity of the method was 0.01 mcg/cm^2 at 500 nm and the method obeyed the Beer law up to 1 mcg/ml. This reagent could be used to determine gold in biological materials after ashing of the samples. Tin(II) chloride has been used to develop a yellow to purple colour with gold with a sensitivity of 0.05 mcg/ml. This reagent has been used to determine gold in excretion material. Acid strength is critical in this method for accuracy and precision. Goodwin (1960) determined gold in urine, plasma or tissue (ashed with perchloric and nitric acid) by oxidation of o-toluidine with auric chloride to produce a stable colour with maximum absorption at 427 nm. The method was sensitive to 1 mcg of gold and followed the Beer law over the range 1-40 mcg. Interference arose from chloride, bromide and nitrosyl chloride. Required sample sizes were 1-3 ml plasma, 10-50 ml urine and 1-2 g tissue. More recently Kothny (1969) reported a procedure for determination of gold in urine, after wet ashing, addition of ethylene glycol monomethyl ether, and ethylene diamine tetraacetic acid, with crystal violet. A toluene extractable complex was read at 605 nm for quantitation with a sensitivity of 0.1 mcg. Walton (1970) determined gold in urine (10 ml) and plasma (1 ml), wet ashed with chloric acid then aqua regia, buffered to pH 3, and with the addition of potassium fluoride, complexation with di-2-pyridyl ketoxime. The colour was read at 433 nm and had a molar absorptivity of 1.89×10^4 and a sensitivity of 0.01 mcg/cm^2. The method was used for concentrations of gold in the range 2.3 to 7 ppm, and had a precision of 0.11 to 0.61% (coefficient of variation). The method had a recovery of 98-101.8% of the gold content of tissues.

Emission spectroscopy can be used for the determination of gold in biological samples. The gold containing sample, 200 microlitres of serum or synovial fluid, is mixed with a known amount of iron as internal standard and 10 microlitres of the mixture is dried at 120°C onto a carbon electrode. The metals are excited by a spark and the emissions are recorded on a photographic plate. Quantitation is done by comparison of the darkening of the film by the gold emission at 242.8 nm and the iron emission at 243.0 nm. The working range of the method was 1-50 mcg/ml with an accuracy of ± 0.5 mcg/ml at the lowest concentrations. Twenty seven serum samples could be analyzed in 4-5 hours (Clausen, 1962a).

Polarography was applied to the determination of gold in 5 to 10 ml of blood or serum which was first wet ashed or dry ashed (550°C), then the gold, as auric chloride, was extracted into ether to separate it from interfering copper and iron, evaporated and a polarogram was run in 2 M potassium hydroxide over the voltage range 0-0.25 V against a calomel electrode. The sensitivity was 1 mcg, and the accuracy was within 2.1% (Christian, 1965). Schmid (1973) described the use of anodic stripping voltammetry for the determination of 0.02 to 2 mcg/ml of gold in serum. Ten ml of the serum was first wet ashed with nitric then chloric acid, then the gold was oxidized with ceric perchlorate. After addition of hydrogen peroxide the gold was electrodeposited on carbon paste electrodes. It was quantitated by stripping at constant current. The method had a precision of 2.5-16% (coefficient of variation) but was unstable and required frequent re-calibration.

Atomic absorption spectroscopy (AAS) has received considerable attention recently for the determination of gold in biological samples because of its advantages of simplicity, speed due to minimal sample preparation, sensitivity, and freedom from interferences. In this method the absorption of light, usually produced by a hollow cathode lamp containing a gold cathode, by atoms of gold is used to measure the concentration of the metal. The most sensitive and most frequently used line is 242.8 nm, although gold also emits light at 267.6 nm, 274.8 nm and 312.3 nm. In most cases atoms of gold are produced by aspiration of a solution of gold into an air/acetylene flame where solvent is evaporated, matrix is combusted and atoms of gold are produced. Sensitivity of flame AAS can be improved by using organic solvents because their combustion in the flame increases the flame temperature and their lower viscosity compared to water increases the aspiration rate and thus increases the concentration of the element in the flame (Dawson, 1967). Three types of interference can occur in AAS: spectral, chemical (either suppression or enhancement) and physical (Dawson, 1967). Spectral interference results from incomplete discrimination between spectral lines of different elements but AAS of gold is relatively free of this because the radiation source produces only gold radiation. Chemical interference is due to the presence in the sample of other chemical substances (usually cations and anions) which alter the efficiency of production of atoms. This can occur if poorly dissociable complexes are present in the sample (eg calcium phosphate). Physical interferences are nonspecific in nature and are usually due to organic material in the sample. For example, protein in the solution, because of its viscosity, may reduce the aspiration rate and thus reduce the amount of sample in the flame. Spectral and chemical interferences have not been found in the determination of gold (Khalifa, 1965). In biological samples usually the only interference problems are physical interferences. Large excesses of platinum and palladium can cause spectral interference (Elwell, 1966) but this would not be a problem in biological samples. Oxalates can precipitate gold from solution (Khalifa, 1965). Gold in serum or plasma is frequently determined by flame AAS after sample dilution to reduce the viscosity (Dietz, 1973; Dunckley, 1971) or by dilution with a surfactant solution again to reduce viscosity (Lorber, 1968a). Gold in synovial fluid may be determined after treatment of the fluid with hyaluronidase and dilution with surfactant (Lorber, 1968a). Sensitivity can be improved somewhat by extraction of gold from the sample with methyl isobutyl ketone (MIBK) after wet ashing (Dunckley, 1973), by oxidation of gold to gold(III) with potassium permanganate and treatment with hydrochloric acid to assure extraction of all forms of gold (Balzacs, 1972), or after complexation with ammonium pyrrolidine dithiocarbamate (Frajola, 1967; Harth, 1973; Philips, 1966). Some workers have first ashed the samples then redissolved them in water (Dunckley, 1971; Tompsett, 1968). Sensitivity, when aspirating aqueous solutions, was 0.1-0.3 mcg/ml (Philips, 1966; Dunckley, 1971; Elwell, 1966; Frajola, 1967) or, when aspirating MIBK solutions, 0.05-0.1 mcg/ml (Dunckley, 1973; Balzacs, 1972; Frajola, 1967). Precision measured as standard deviation was reported as 0.06-0.2 mcg/ml (Balzacs, 1972; Lorber, 1968) or, measured as coefficient of variation, as 1-16% (Dunckley, 1973; Harth, 1973; Mascarenhas, 1972). Usually at least 1 ml of solution must be aspirated for a single determination so when sample size is limited and concentration is near the sensitivity limit there can be a problem.

Atomic absorption spectroscopy, with production of atoms by means of electrical heat rather than a flame, offers greater sensitivity but requires smaller samples. Because of the latter requirement concentration of samples may be needed for optimal sensitivity with dilute samples. Agett (1971) investigated flameless AAS using a carbon filament to atomize the gold in an atmosphere of argon. Attenuation of the gold signal by thousand fold excesses of cerium, copper, chromium, iron, manganese, molybdenum, nickel, palladium or tungsten occurred. The interference was eliminated by restricting the viewing field used. Sensitivity was 2×10^{-10} g. Later the method was applied to 2 microlitre serum samples (Agett, 1973). No sample preparation was required; the instrument was operated in a four cycle mode: dry at a relatively low temperature, ash organic material in two steps at two different temperatures and analyze at the highest temperature. The precision of the method was ±4% (coefficient of variation). After considering the differences in sample size required for flame or flameless AAS, Agett (1973) concluded that the flameless technique was 20 times more sensitive. Agett (1971) achieved better sensitivity (5×10^{-12} g at 242.8 nm and 4×10^{-12} at 267.5 nm) using atomic fluorescence; however no application to biological samples was made.

Radioactive tracers of gold have been used to study the distribution of gold and to calculate the gold concentration in tissues by simple isotope dilutions. Gold-198 is most commonly used (Lawrence, 1961; McQueen, 1969; Oka, 1973; Smith, 1973; Swartz, 1960; Tonna, 1963). It has a half life of 2.7 days and decays by beta and gamma emission. The 0.411 MeV gamma is used for detection. Counting tissues and organs of patients given radioactive gold permits studies of gold distribution in humans (Oka, 1973; Lawrence, 1961). Because of the beta component of its decay, autoradiography is possible (Tonna, 1963). The energy of the beta is 0.962 MeV so this results in poor resolution. The relatively short half life of gold compared to the long time which gold remains in the body makes long term studies using ^{198}Au impossible. Gold-195 which has a half life of 183 days and decays by gamma emission partially overcomes this problem. The 98.5 KeV gamma is detected. Gottlieb (1972b), Gerber (1972a,c) and Jeffrey (1958) used this longer half life isotope of gold . When gold has already been administered to the subject prior to the isotope, gold levels calculated from counting radioactivity may not accurately reflect gold levels in slowly equilibrating compartments of the body which may exist for gold. Studies by Gerber (1972a) and Gottlieb (1971b) showed that gold-195 and stable gold in body fluid did equilibrate freely. Of the other isotopes of gold, only ^{196}Au (half life 6.18 days) and ^{199}Au (half life 3.15 days) have sufficiently long half lives to be useful. They offer no advantage over ^{195}Au.

Activation analysis, where a sample is irradiated with nuclear particles such that some of the atoms of the sample will interact with the nuclear particle and be converted into different isotopes, is very useful for the analysis of gold in biological materials. Several nuclear reactions can be used to activate gold. The cross section of the nuclear reaction which is a measure of the probability that the nuclear particle will interact with the nucleus of the atom being irradiated, also gives a measure of the sensitivity of the reaction for activation analysis. The following reactions are possible:

^{197}Au(n,gamma)^{198}Au - 98 barns, thermal neutrons

^{197}Au(n,gamma)^{198}Au - 1558 barns, epithermal neutrons

^{197}Au(n,2n)^{196} Au - 0.142 or 2.6 barns, 14 MeV neutrons

^{197}Au(n,p)^{197}Pt - 0.0024 or 0.020 barns, 14 MeV neutrons

^{197}Au(n,alpha)^{194}Ir - 0.00043 or 0.0005 barns, 14 MeV neutrons

(Texas Nuclear, 1964; Gillespie, 1961; Lobandov, 1966). The most sensitive reaction for gold is the n,gamma reaction producing ^{198}Au. A possible interfering reaction is ^{198}Hg(n,p)^{198}Au but this is negligible in biological samples (Brune, 1966). A more important source of interference in biological samples is the production of large amounts of ^{24}Na which results in a large background even when high resolution detectors are used, such as the Ge(Li) detector. The use of epithermal neutrons results in a decrease in this interference. This is because the resonance integral for ^{197}Au is 1558 barns compared to its thermal neutron cross section of 98 barns, giving a ratio of 15.8, while the resonance integral/ thermal neutron cross section for ^{23}Na is 0.5 (Lobandov, 1962). When the sample is irradiated in a cadmium shell, thermal neutrons up to 0.4 eV are absorbed and activation is due to epithermal neutrons. Debons (1962) in studying gold levels in aurothioglucose treated mice did not find the use of cadmium shells necessary. Sensitivity of neutron activation analyis (NAA) depends on the flux of neutrons used in the activation process. Typically 10^{12} n/cm^2-sec are available in reactors which will give sensitivities of 10^{-11} g gold with irradiation to saturation (Rakovic, 1970). When irradiating a simple mixture of two elements, the element of interest producing an isotope of short half life and the other element producing an isotope of long half life, short irradiation periods will make the activity of the element of interest much greater than the activity of the interfering isotope. In the opposite case where the long half life isotope is the element of interest it would be better to irradiate for a long time till the short half life isotope is saturated and the long half life isotope is still approaching saturation, then allow a decay period for the short half life isotope to decay away. In a complex mixture, such as blood, there are many elements which could be activated and the optimal irradiation and decay time for the highest ratio of ^{198}Au counts to interfering counts is a complex function of all the elements present. Plantin (1965) published data on the activity of trace elements in 1 ml of whole blood irradiated with 3.4×10^{13} n/cm^2-sec of thermal neutrons. Using this data the optimal irradiation and decay time required for best detection of gold has been calculated using a Focal program (Appendix I). Using exponential activation and decay equations and considering only gamma emitting isotopes with energies greater than ^{198}Au (0.411 MeV) the optimal irradiation and decay time for 0.1 ml of blood irradiated with 2×10^{13} n/cm^2-sec was determined to be 12.5 days (294 hours) irradiation and 7.75 days (186 hours) decay. This should give 1600 dpm due to gold in blood of a normal individual who has never received gold therapy. Normal gold levels in blood are 10^{-11} to 10^{-9} g/g. Examination of procedures applied to NAA of gold in biological samples reveals that optimal irradiation and decay times have not been used, thus reported sensitivities are not the maximum attainable. Activation times of 0.5 to 100 hours at neutron fluxes of 5×10^{11} to 2×10^{13} n/cm^2-sec with decay times of 20 hours to 18 days have been used, achieving sensitivities of

$2.7x10^{-9}$ to $5x10^{-8}$ g/g of sample (Babb, 1967; Botzvadze, 1969; Brune, 1966; Debons, 1962; Helby, 1962; Kanabrocki, 1965; Parr, 1963; Rubin, 1967; Solvsten, 1964; Tobias, 1949). Both nondestructive NAA, where there is no radiochemical separation of ^{198}Au after irradiation (Babb, 1967; Debons, 1962; Helby, 1962; Kanabrocki, 1965; Rubin, 1967) and destructive NAA have been employed. The latter used ion exchange resins (Botzvadze, 1969; Brune, 1966; Gibbons, 1958), chemical separations (Parr, 1963; Solvsten, 1964), or precipitation of gold (Gibbons, 1958). Precision of the NAA method for gold applied to biological samples has been reported as 2-10% (coefficient of variation) (Gottlieb, 1972b; Rubin, 1967; Solvsten, 1964).

Neutrons are not the only means of inducing radioactivity; high energy gamma rays and protons can also be used (Wainerdi, 1965). ^{197}Au(gamma,gamma)^{197}Au reaction has a sensitivity of 0.1 mg, the metastable gold has a half life of 7.5 seconds. ^{197}Au(gamma,n)^{196}Au reaction has a sensitivity of 1.2 nanograms; the ^{196}Au has a half life of 6 days (Lenihan, 1972). X-ray and gamma ray spectrography can be used to analyze gold with sensitivities for gold of 21 mcg/g (Burkhalter, 1970; Earle, 1969). Application to the analysis of gold in biological samples has not been reported. Excitation can also be produced by ions, electrons, and laser microprobes in the determination of gold in biological samples (Tretyl, 1972). These techniques are suited to localization of gold within tissues since small samples are used. The ion microprobe uses a 10^{-11} g sample and achieves a sensitivity of $10\text{-}10^{-2}$ ppm. The electron microprobe uses a 10^{-12}g sample and has a sensitivity of $10^{3}\text{-}10$ ppm. The laser microprobe requires a 10^{-8}g sample and has a sensitivity of $10^{2}\text{-}10^{-1}$ ppm.

Histochemical techniques have been used for localization of gold in tissues. Elftman (1945) used a technique which reduced gold to rose colloidal gold in the tissue with stannous chloride. Querido (1947) developed a photochemical technique for the localization of gold in tissue. Electron microscopy can be used to localize gold in tissues if it is deposited in colloidal particles or in sufficiently large aggregates. Gold dissolved in tissues cannot be localized by electron microscopy because atoms cannot be resolved.

It can be seen that all the methods for the analysis of gold determine gold as an element and not the intact gold compound. Perhaps chromatography of gold compounds could be useful in the analysis of intact or altered chemical forms of gold in the body. Chromatography of thiourea and dithiozonate complexes of gold has been reported (Marcotrigiano, 1972; Seiler, 1969). Table 4 summarizes the sensitivity of various methods of gold analysis.

TABLE 4 METHODS FOR ANALYSIS OF GOLD AND THEIR SENSITIVITIES

Method	Absolute sensitivity g	Relative sensitivity g/g or g/ml
Gravimetric	2x10^{-5}	
Absorption spectroscopy	1x10^{-7}	2-5x10^{-8}
Emission Spectroscopy		6x10^{-6}
Polarography	10^{-6}	
AAS flame		0.5-3x10^{-7}
AAS flameless	2x10^{-9}	
Atomic fluorescence[1]	4x10^{-12}	
NAA (n,gamma)	10^{-11}	0.26-5x10^{-8}
Gamma Spectrography[1]		
(gamma,n)	1.2x10^{-9}	
(gamma,gamma)	10x10^{-4}	
Ion microprobe	10^{-17}-10^{-19}	10^{-8}-10^{-5}
Electron microprobe	10^{-15}-10^{-17}	10^{-5}-10^{-3}
Laser microprobe	10^{-12}-10^{-15}	10^{-7}-10^{-4}

[1] no reports of application to biological samples.

STATEMENT OF PROBLEM

The review of the possible modes of action for gold indicates that the mechanism of action of gold is still not known. Investigations of the possible role of endogenous corticosteroids in the action of gold are incomplete. Clinical impressions suggest that patients who show toxicity to gold show a greater incidence of remission of rheumatoid arthritis than do those who do not develop toxicity (Krusius, 1970; Bayles, 1956; Freyberg, 1966; Copeman, 1937); however statistical evidence contrary to this view has appeared (Empire Rheumatism Council, 1961b; Hartfall, 1937; Gottlieb, 1972b). Toxicity may bring about improvement by stimulation of the adrenal cortex leading to increased endogenous levels of corticosteroids. Secondly since gold is accumulated by the reticulo-endothelial cells of the adrenal, cells which have a predominant role in 17-hydroxylation of steroids, gold could act to stimulate biosynthesis of steroids in these cells. Studies in rats, animals which are predominantly corticosterone secretors, would not detect this type of stimulation of corticosteroid biosynthesis. Man is predominantly a cortisol secreting animal so stimulation of the biosynthesis of cortisol by gold could occur in man even when it was undetected in rats. Studies in man were largely confined to the determination of the urinary excretion of 17-ketosteroids, which is not a good index of adrenal activity since 17-ketosteroids are of both adrenal and gonadal origin and their excretion depends on liver function, for metabolism of 17-hydroxycorticosteroids to 17-ketosteroids and on kidney function for excretion. The active level of corticosteroid in the body depends on many factors: the biosynthesis and secretion of corticosteroids by the adrenal, the secretion of ACTH by the anterior pituitary, the secretion of corticotrophin releasing factor by the hypothalamus, the metabolism of corticosteroids at hepatic and extrahepatic sites, excretion by the kidney, protein binding of corticosteroids, and the circulating levels of corticosteroids. Feedback control mechanisms depend on the level of unbound corticosteroid circulating in the blood, so changes at any level in the system, uncompensated by homeostasis or some other mechanisms, will be reflected by changes in circulating corticosteroid levels.

The approach taken in this study was to determine circulating levels of cortisol and also to determine protein binding parameters for circulating cortisol in various human subjects. Because gold is slow in exerting its therapeutic effect, it is necessary to have gold treated patients at various stages of gold therapy. The study group consisted of patients, chiefly with rheumatoid arthritis. The patients fell into two groups: one group receiving gold therapy, the other group not receiving gold therapy. A third group of 'normal' individuals was used to indicate that the methods of analysis gave reasonable values for normal subjects. This is not strictly a control group because of differences between ages of this third group and the disease groups; however the purpose of this investigation was to study the effect of gold therapy. The effect of the disease, rheumatoid arthritis on corticosteroids has been thoroughly investigated, as is apparent from the introduction. Plasma cortisol levels and cortisol binding were compared in both the gold and non-gold disease groups. Also cortisol levels and binding were stratified according to stage of gold therapy, as measured by plasma gold levels, in an attempt to determine at which stage of gold therapy, gold therapy affected cortisol, if an effect occurred at all. Within the gold group were some patients who were newly initiated to gold; in these patients, who were used as their own controls, cortisol levels and binding were compared before and during gold therapy. In order to complete this project, methodology for the determination of plasma cortisol, cortisol plasma binding parameters and plasma gold had to be worked out or adapted from existing methodology. Secondary to the main purpose of this project, information was available on the relationship between adverse reactions and plasma cortisol and gold levels. Information was also available on several factors affecting plasma gold levels.

The second phase of this work was an investigation of chrysiasis, a rare long term side effect of gold therapy, which results in a permanent alteration of pigmentation of the skin in light exposed areas of the body such that the skin has a bluish to gray hue. Previous investigations of chrysiasis have demonstrated that gold particles are present in the skin but no quantitation of gold in skin of patients with chyrsiasis has been made. Chrysiasis is a dose related phenomenon according to the literature. Skin, hair, nail and blood gold levels were determined in 3 patients exhibiting chrysiasis and in 7 patients not exhibiting chrysiasis but who had been treated with gold. Attempts were made to correlate gold dose with skin gold levels. In order to complete this part of the study highly sensitive methods for analysis of gold in small samples were required.

METHODOLOGY

A. MATERIALS

(i) CHEMICALS

Cortisol, British Drug Houses, Toronto, Ontario; recrystallized from alcohol

Cortisol-1,2-T (45-55 Ci/mM) in 9:1 ethanol:benzene, Amersham Searle, Des Plaines, Illinois. The radiocompound purity of isotopes was checked by thin layer chromatography on silica gel using the following solvent systems: chloroform : absolute ethanol (9 : 1), chloroform : absolute ethanol (7 : 2), chloroform : acetone (2 : 8). Autoradiography of the chromatograms revealed only one spot. The radioactive cortisol was stored at -10°C, but was not frozen because of the ethanol in the solution. All binding experiments were done using ^{3}H-cortisol of specific activity 45 Ci/mM.

2,5-Diphenyloxazol (PPO), scintillation grade, Kent Chemicals Ltd, Vancouver, British Columbia

1,4-Di-[2(5-phenyloxazolyl)]-benzene (POPOP), scintillation grade, Kent Chemicals Ltd, Vancouver, British Columbia

Fuller's earth, technical grade, Fisher Scientific Ltd. Pittsburgh, Pennslyvania

Gold chloride ($HAuCl_4.3H_2O$), Baker reagent grade, Phillipsburg, New Jersey, analysis 49.1% gold

Gold powder, Alfa Inorganics, Beverly, Massachusetts, Lot 10570 analysis 99.9999% gold

Hexafluoroacetylacetone (hfaa), Pierce Chemical Company, Rockford, Illinois

Sodium aurothiomalate, Poulenc Ltd, Montreal, Quebec, Lot 10548, analysis 42.34% gold

Sodium aurothiomalate injection, 25 mg/ml, Poulenc Ltd, Montreal, Quebec, Lot 16, analysis 52.48% gold

Trifluoroacetylacetone (tfaa), Pierce Company, Rockford, Illinois

Toluene tritium calibrated standard, New England Nuclear, Dorval, Quebec, NES-004

Water, demineralized and distilled in glass distillation apparatus

Drugs used in the interference studies were pure substances except as indicated below.

D-Propoxyphene, Lilly, capsule

Prednisone, Elliot-Marion, tablet

Methylprednisolone acetate, Upjohn, Suspension

Betamethasone, Schering, suspension

Indomethacin, Merck Sharp and Dohme, tablet

Amitriptyline hydrochloride, Merck Sharp and Dohme, tablet

Diazepam, Horner, tablet

Chlordiazepoxide hydrochloride, Horner, capsule

Primidone, Ayerst, tablet

Propantheline bromide, Searle, tablet

Orphenadrine citrate, Riker, tablet

All other chemicals were reagent grade or better.

The following solutions were used.

Brays' Solution

Naphthalene	60g
PPO	4 g
POPOP	0.2 g
Methanol	100 ml
Ethylene glycol	20 ml
Dioxane to	1 litre

Ringer's Solution

Sodium chloride	0.65 g
Potassium chloride	0.014 g
Calcium chloride	0.012 g
Sodium bicarbonate	0.020 g
Sodium acid phosphate	0.001 g
Distilled water to	1 litre

(ii) BLOOD SAMPLES

Blank plasma was plasma taken from persons who had not received gold therapy. Except where indicated otherwise, venous blood samples were taken into citrated 'Vacutainers', Becton-Dickinson and Company, Columbus, Nebraska. Each 'Vacutainer' contained 0.5 ml of 3.8% sodium citrate as anticoagulant. The volume of each blood sample was measured so that correction for dilution by the citrate solution could be made. Blood samples were centrifuged within 2 hours of sampling to separate red blood cells. Plasma was usually stored at -10°C. In some cases, where specifically indicated, it was stored at 5°C or used fresh. Red cells and clot were stored at 5°C.

(iii) INSTRUMENTS

Atomic absorption spectrophotometer, Perkin-Elmer, Norwalk, Conneticut, Model 290B, equipped with a 2 inch single slot burner, a Beckman 10 inch recorder Model 1005. Operating conditions: wavelength = 242.8 nm, oxidant air 14 units 40 psig, fuel acetylene 14.26 units 8 psig.

Atomic absorption spectrophotometer, Perkin-Elmer, Model 305B, equipped with deuterium background correction, HGA-2000 carbon tube furnace, Beckman 10 inch recorder. Operating conditions: wavelength = 242.8 nm, purge gas nitrogen 7 units 40 psig, dry 30 seconds at 200°C, char 30 seconds at 900°C, atomize 7.5 seconds at 2500°C, sample size= 50 microlitres.

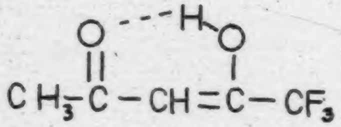
Liquimat 220, Picker Nuclear, White Plains New York, equipped with cesium-137 external standard. Instrument settings: A 10-350, C 250-450, D 450-900, calibrate at 500. Quench correction was done using the external standard channels ratio method using a set of quenched standards (5 or 10 ml total volume) prepared using Bray's solution as solvent, carbon tetrachloride as quencher, and tritiated toluene as standard.

PDP 8/L and PDP-1105 computers, Digital Equipment Corporation, Maynard, Massachusetts, language Focal.

B. GOLD ANALYSIS

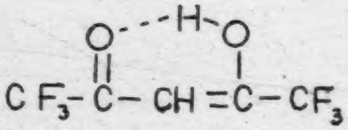
(i) GOLD CHELATES

Volatile derivatives of some metals have been prepared by chelation of the metal with fluorinated beta-diketones (eg trifluoroacetylacetone (I) hexafluoroacetylacetone (II), Ross, 1970).



$$\mathrm{CH_3-\overset{\overset{O}{\|}}{C}-CH=\overset{\overset{O-H}{|}}{C}-CF_3}$$

I



$$\mathrm{CF_3-\overset{\overset{O}{\|}}{C}-CH=\overset{\overset{O-H}{|}}{C}-CF_3}$$

II

If a volatile derivative of a metal can be prepared it may be possible to determine the metal quantitatively by gas liquid chromatography. Use of electron capture detection should give high sensitivity ($2x10^{-14}$ g reported for chromium) (Ross, 1970). Aluminum, beryllium, cobalt, copper, chromium, gallium, hafnium, iron, indium, lithium, magnesium, manganeses, nickel, palladium, platinum, rhodium, scandium, strontium, thorium, titanium, uranium, yttrium, zinc and zirconium have been chromatographed as metal chelates (Ross, 1970; Taylor, 1971; Sieck, 1971; Bayer, 1971). Preparation of gold chelates of this type has not been reported, so attempts were made to prepare tfaa and hfaa gold chelates with a view to using them for quantitation of gold by gas chromatography. In order to be useful analytically, the reaction between gold and the chelator must be quantitative and the chelate formed must be sufficiently volatile and stable to be amenable to gas chromatography.

A. To 0.001M gold metal (dissolved in aqua regia) was added 0.0026M tfaa (aqueous suspension); after the addition of ammonium hydroxide a yellow precipitate formed which was positive for gold[1], but was insoluble in benzene and acetone.

B. To 0.001M gold metal (dissolved in aqua regia) was added 0.0026M tfaa (in 40% aqueous ethanol); after the addition of ammonium hydroxide a brown precipitate formed which was positive for gold, insoluble in benzene, or hydrochloric acid-benzene, but soluble in hydrochloric acid-ethanol. The precipitate exhibited no absorption in infrared region. The remaining aqueous solution was also positive for gold.

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[1] Test for gold using rhodamine B (0.5% aqueous solution): one drop test solution + one drop hydrochloric acid + one drop rhodamine B, shake with six to eight drops of benzene. If gold is present, the benzene layer turns red-violet to pink, and after one minute under quartz lamp displays orange fluorescence. Limit of detection 0.1 micrograms (Feigl,1958).

C. Auric chloride, 0.001M (dissolved in water and neutralized with sodium bicarbonate) was heated under reflux with tfaa, 0.004M. A brown green precipitate formed which was positive for gold, insoluble in water, or acetone, and exhibited no absorption in infrared. When the aqueous phase was extracted with benzene it yielded a brown oil (positive for gold, absorption in infrared notably 1710(s) and 1620(w) cm^{-1}. The carbonyl absorption of tfaa occurs at 1600 cm^{-1}, which is characteristic of C=O absorption for beta-diketones in the enolic form (Dyer,1965). Belford (1956) reported that copper chelates of these type of beta-diketones have absorptions at lower frequencies than the ligand. This suggests that some chelate was obtained; however the reaction and extraction was not complete because the remaining aqueous phase was still positive for gold. The elemental analysis (C 45.8%, H 5.3%) did no correspond to any formulae proposed for the chelate [$(C_5H_4O_2F_3)_3Au$ requires C 27.4%, H 1.8%; $C_5H_4O_2F_3Au$ requires C 17.3% H 1.1%].

D. Auric chloride (C.985mM) was heated under reflux with tfaa (5mM) for two hours. A precipitate formed which was positive for gold and which had a weak infrared absorption in the carbonyl region. Distillation yielded only tfaa (confirmed by infrared) so no volatile derivative of gold had been formed.

E. Auric chloride (0.986 mM) was heated under reflux with hfaa (5 mM, dehydrated with 36 N sulfuric acid). A precipitate which was soluble in acetone was formed. The precipitate decomposed on heating. A solid which sublimed on the refluxing apparatus was shown by infrared to be hfaa dihydrate.

F. Auric chloride (0.03M, neutralized with 0.2 M sodium hydroxide and buffered in the range pH 1.2 to 13) was extracted with 10 volumes of tfaa in benzene (0.1 M). Gold in each phase was determined by atomic absorption spectroscopy. The aqueous phase was diluted 1 to 50 then aspirated into the atomic absorption apparatus, while a 0.2 ml aliquot of the benzene phase was evaporated to dryness then dissolved in 0.2 ml of aqua regia which was subsequently diluted to 10 ml with distilled water and aspirated. The greatest extraction of gold was at pH 7 (Table 5). When gold chloride was extracted with benzene lacking tfaa no gold was found in the benzene phase. In a similar series with hfaa (dehydrated with sulfuric acid) covering pH 1.2 to 6 no gold could be extracted into the benzene phase.

It appears that gold does form a complex with tfaa; however the low yield (13.92% at pH 7) makes this approach unsuitable for quantitative analytical work; therefore the preparation of gold chelates was not pursued further.

TABLE 5 EFFECT OF pH ON EXTRACTION OF GOLD BY 0.1 M TRIFLUOROACETYLACETONE IN BENZENE

pH	% Gold in benzene phase
1.2	3.39
2	2.11
3	1.06
4	1.06
5	3.39
6	4.65
7	13.92
8	11.40
9	6.35
10	5.50
11	2.11
12	2.11
13	1.06

(ii) ATOMIC ABSORPTION SPECTROSCOPY

a. ANALYSIS OF GOLD IN PLASMA SAMPLES BY FLAME ATOMIC ABSORPTION SPECTROSCOPY

The method used was essentially that of Lorber (1968a) with modifications as described below. Standard curves covering the range 0-20 mcg/ml of gold were prepared each day using freshly prepared gold standards because of the instability of dilute gold solutions (Beamish, 1961). The standards were prepared in one of two ways: (a) dilution of gold chloride with demineralized distilled water to give aqueous standards of the required concentration, (b) dilution of gold chloride with a 1:1 mixture of blank plasma and 2% sodium lauryl sulfate to give plasma standards of the required concentration. A typical aqueous standard curve is given in Figure 8 and a typical plasma standard curve is given in Figure 9. It will be noted that the slope of the plasma standard curve is less than that of the aqueous standard curve indicating lesser response for a given gold concentration in diluted plasma compared to water. This is likely due to a slower aspiration rate for plasma because the diluted plasma has a higher viscosity than water. Dilution of plasma with an equal volume of 2% sodium lauryl sulfate reduces the viscosity of plasma sufficiently to allow efficient aspiration but not sufficiently to equal the aspiration rate for water. It will also be noted that the Y intercept of the plasma standard curve is not equal to zero, indicating a blank absorption for plasma. When diluted plasma containing no gold was aspirated 1-2% response was observed but this could not be measured accurately. The Y intercept of the plasma standard curve gives an estimate of the blank response due to plasma.

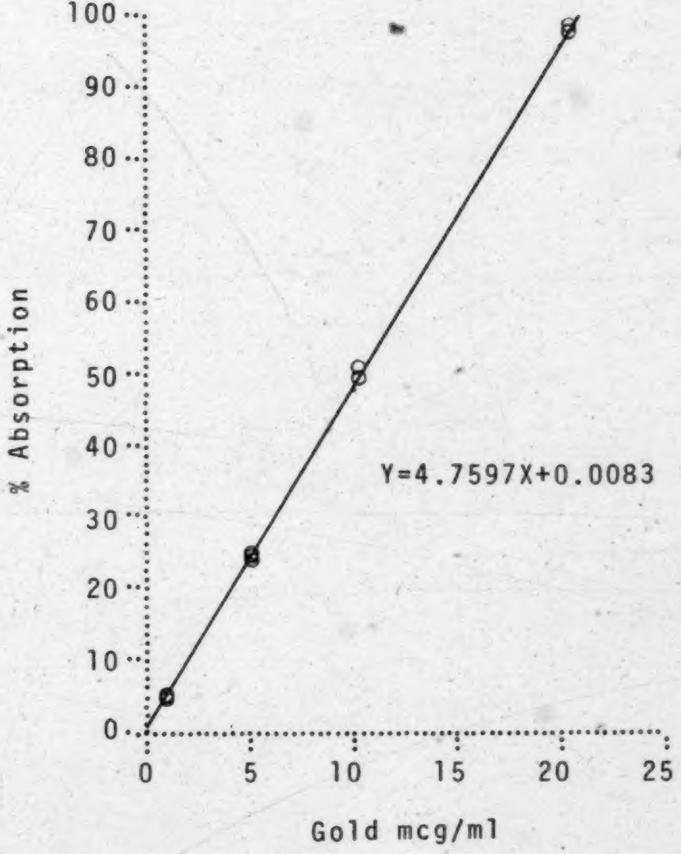


FIGURE 8 Gold Standard Curve - Flame Atomic Absorption Spectroscopy Using Gold Chloride in Aqueous Solution as Standard.

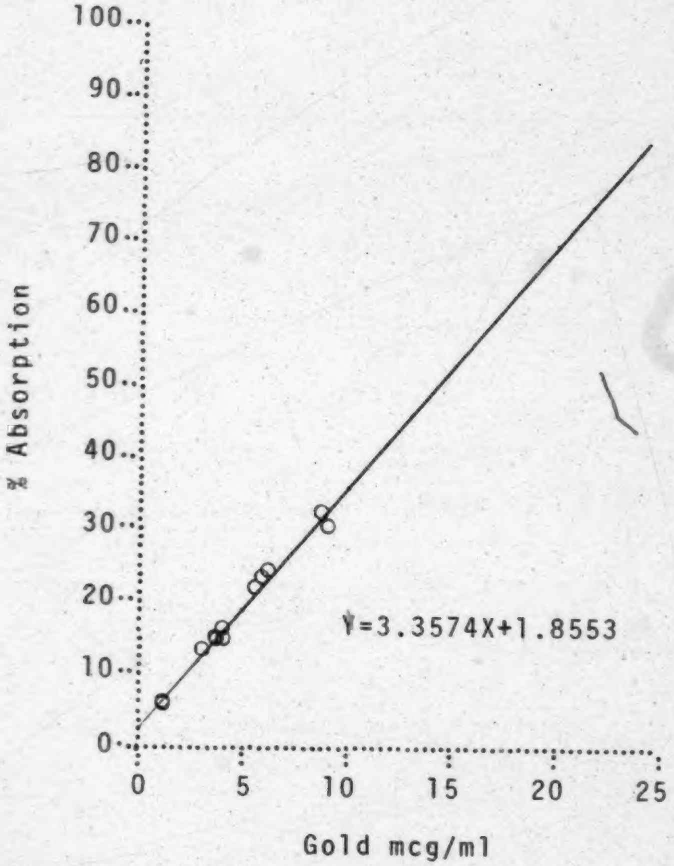


FIGURE 9 Gold Standard Curve - Flame Atomic Absorption Spectroscopy Using Gold Chloride and Gold Sodium Thiomalate in Plasma as Standard.

These differences in the plasma and aqueous standard curves indicate that correction for plasma blank and lower efficiency of detecting gold in plasma must be made when plasma samples are compared to an aqueous standard curve. A group of plasma samples were assayed in comparison to an aqueous curve after correction for blank by subtraction of 1.9% absorption from the observed % absorption. The gold concentration obtained in this manner was compared to the known gold concentration to obtain the % recovery of gold. The average recovery was 84% (Table 6).

TABLE 6 RECOVERY OF GOLD IN PLASMA AFTER CORRECTION FOR APPARENT BLANK[1]

Type of Sample	Gold concentration mcg/ml	% recovery
a Gold sodium thiomalate	2.5	92.2
	2.5	82.8
	2.5	82.8
	6.25	89.8
	7.5	87.2
	7.5	87.2
	7.5	84.1
	11.25	85.3
	12.5	86.2
	17.5	83.1
b Gold chloride + Gold sodium thiomalate	8.16	84.1
	8.16	77.3
	8.16	74.4
	11.91	86.5
	18.16	74.9

[1] Observed % absorption was corrected for blank of 1.9, then gold concentration was calculated using a calibration curve derived from gold choride standards. Subsequent determinations of plasma standard curve gave a similar value suggesting that the blank value is constant. Mean ± standard deviation for a = 86.0 ± 3.1, for a and b 84.0 ± 5.1.

A group of plasma samples were prepared at known concentration by addition of gold to blank plasma. These samples were assayed using a plasma standard curve and also using an aqueous standard with subtraction of the blank (1.9%) from the absorption for each sample, and correction of gold concentration from standard curve for recovery (84%). Table 7 shows that the two methods gave comparable results for gold content of plasma samples.

TABLE 7 COMPARISON OF SINGLE RECOVERY CORRECTION FACTOR AND AQUEOUS STANDARD CURVE TO NO RECOVERY CORRECTION AND PLASMA STANDARD CURVE

Sample concentration mcg/ml	% of correct value aqueous standard curve, single recovery factor	Plasma standard curve, no recovery factor
2.5	109.8	93.1
7.5	103.8	104.5
8.16	88.6	88.6
11.25	101.6	105.1
17.5	98.9	104.3
mean	100.5	99.1
s	7.8	7.7

The chemical form of gold in the plasma of patients treated with gold sodium thiomalate is not known; therefore it was of interest to compare the response for gold in ionic form (gold chloride) and in covalently bonded form (gold sodium thiomalate). Standard curves prepared using these two forms of gold were not significantly different (Figure 10); since F calculated for the variance about regression for each form of gold was not significant.

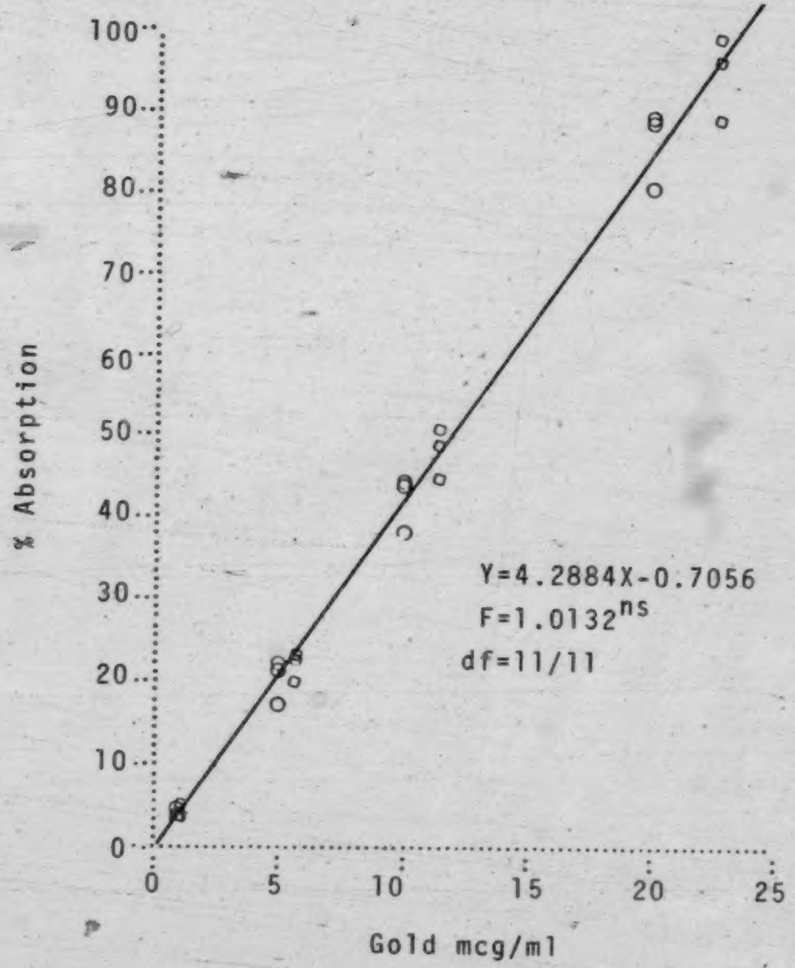


FIGURE 10 Gold Standard Curve - Flame Atomic Absorption Spectroscopy Showing no Difference Between Gold Chloride (○) or Gold Sodium Thiomalate (○).

For the assay of gold in plasma samples, one ml (as little as 0.5 ml could be used) of plasma was diluted with an equal volume of 2% sodium lauryl sulfate and aspirated into the flame. A set of standards were measured several times during any given assay. The concentration of gold in the sample was determined by comparison with the standard curve. A program (Appendix I) was used to calculate by regression the equation of the standard curve, to interpolate the gold concentration for the samples, and to correct for dilution. This method of determining gold is rapid, allowing 36 samples to be run in a half day. Clogging of the burner slot may occur after aspiration of 24 plasma containing solutions. When this occurs it is necessary to shut off the flame and clean the slot with a razor blade. If the burner slot is kept clean in this way, there is little change in sensitivity during an assay.

Precision of the assay, expressed as standard deviation of replicate assays of a plasma sample containing 4 mcg/ml of gold, was 0.59 mcg/ml (coefficient of variation = 13.8%). The sensitivity, defined as the concentration of gold giving 1% absorption, was 0.21 mcg/ml for aqueous standard curves and 0.26 mcg/ml for plasma standard curves. These values were obtained by taking the reciprocal of the slope of the standard curve. Because plasma samples were diluted with an equal volume of sodium lauryl sulfate solution prior to assay, the sensitivity for aqueous and plasma samples was actually 0.42 mcg/ml and 0.52 mcg/ml, respectively. The noise level was usually 1-2% absorption. No improvement in sensitivity was obtained by scale expansion of the output signal because the noise level also increased.

b. ANALYSIS OF GOLD IN TISSUE AND BLOOD BY FLAMELESS ATOMIC ABSORPTION SPECTROSCOPY

The Perkin-Elmer 305B atomic absorption spectrophotometer equipped with an HGA-2000 carbon tube furnace was used to assay gold in skin, hair, fingernails, plasma, serum, red blood cells or clot. It was found to be necessary to wash all glassware in aqua regia then rinse it with glass distilled water . Standard curves covering the range 50-5000 picograms (pg) of gold were prepared (Figure 11) with no scale expansion and covering the range 5-1250 pg gold with scale expansion 3 (Figure 12). The sensitivity was 49 pg with no scale expansion and 16 pg with x3 scale expansion. The noise level was very low; it was virtually absent with no scale expansion and was less than 0.25% absorption with x3 scale expansion. Fifty microlitre volumes of samples and standards were always used. Hair and nail specimens were washed with distilled water to remove possible surface contamination. Samples of red blood cells, clot, skin, hair or nails were weighed. A measured volume (0.1 ml) of plasma or serum was weighed. The organic material was digested with concentrated nitric acid (0.1 ml/ml final dilution) by gently heating (45°C) overnight. The samples were then diluted with distilled water to a known volume such that for tissue samples the dilution was about 1 mg tissue/ml of solution and for blood specimens about 10 mg/ml solution. Fifty microlitre aliquots of this final dilution were assayed by flameless atomic absorption spectroscopy at either no or x3 scale expansion depending on the gold content of the tissue. Several replicate analyses were made for each sample. Gold content of aliquots were calcuated from the equation of the standard curve. The actual sample gold concentration was obtained by correction for dilution.

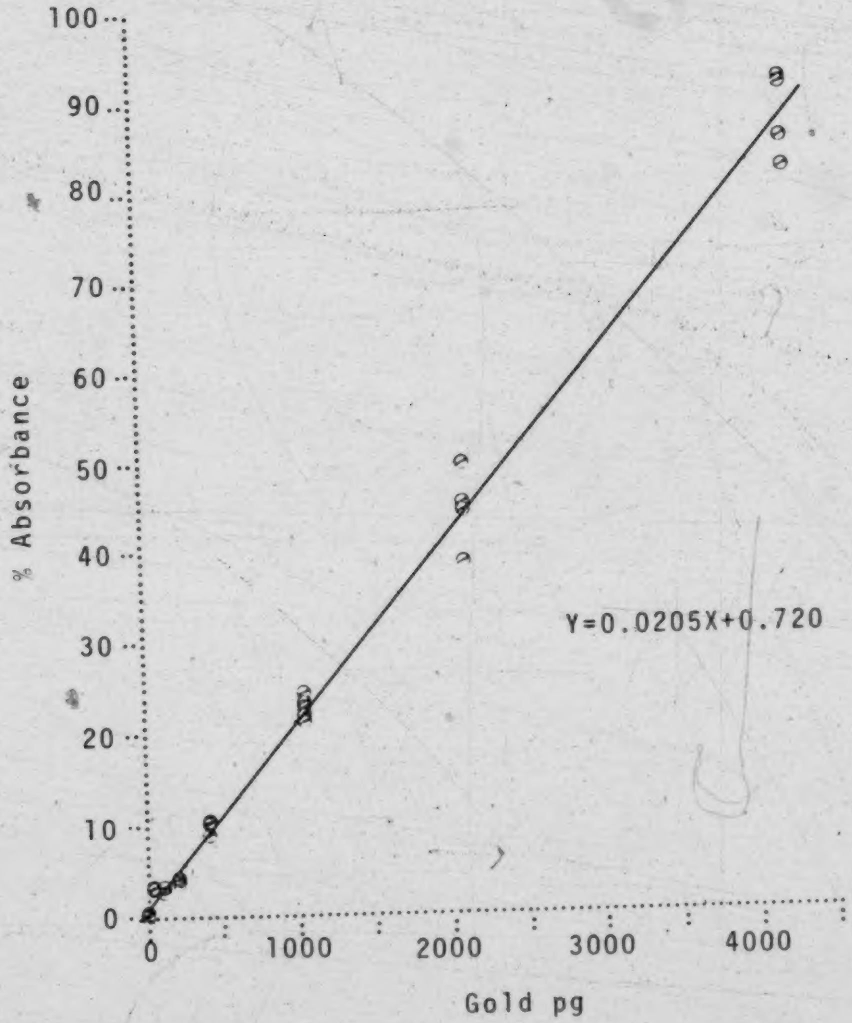


FIGURE 11 Gold Standard Curve - Flameless Atomic Absorption Spectroscopy.

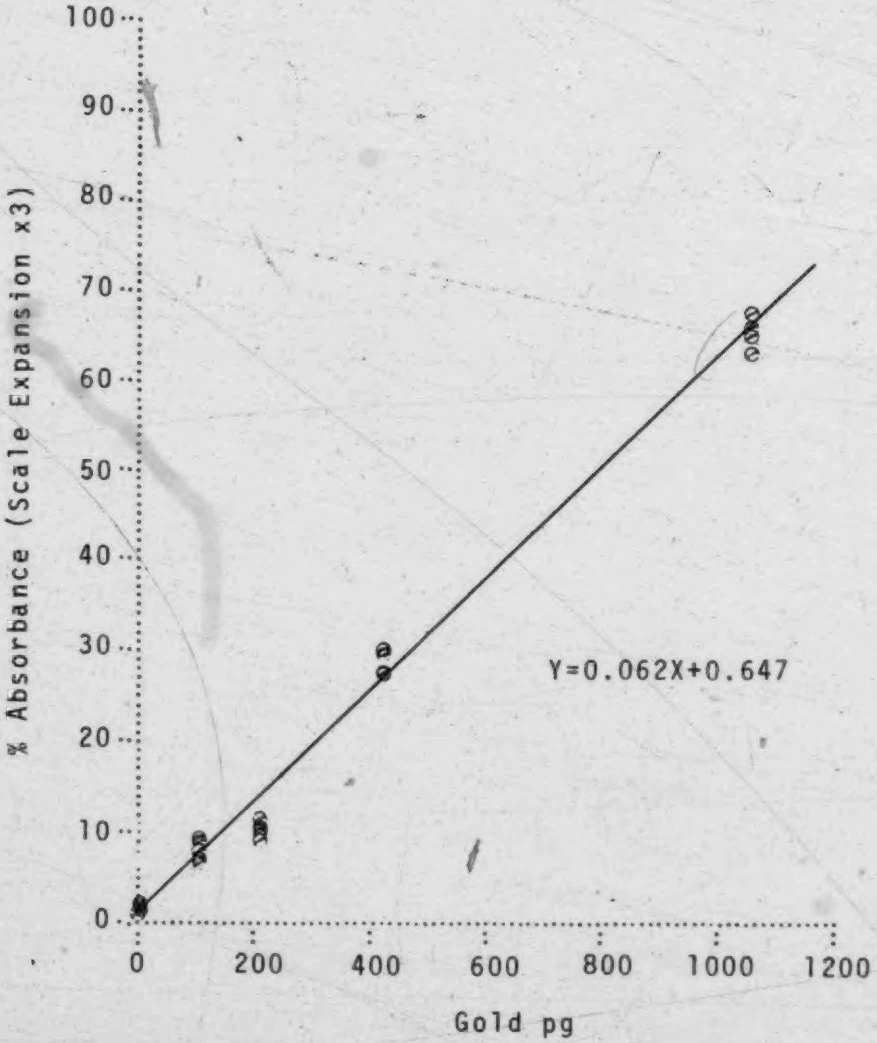


FIGURE 12 Gold Standard Curve - Flameless Atomic Absorption Spectroscopy, Scale Expansion x3.

Precision of the assay expressed as standard deviation of duplicate analyses for a given dilution was 0.06 mcg/g (number of duplicates = 52, concentration range 0-10 mcg/g), 1.873 mcg/g (n=7, concentration range 10-20 mcg/g), 1.816 mcg/g (n=4, concentration range 30-60 mcg/g), 1.498 mcg/g (n=2, concentration range 60-90 mcg/g), and 11.884 mcg/g (n=2, concentration range 120-150 mcg/g).

Plasma, hair, fingernail and skin from normal individuals who had not received gold therapy gave small responses when processed in the manner described above (Table 8). The absorption was not related to the concentration of tissue in the solution, therefore this is not a matrix effect. Distilled water or diluted nitric acid gave no absorption.

TABLE 8 RESULTS OBTAINED BY FLAMELESS ATOMIC ABSORPTION FOR SAMPLES FROM NORMAL INDIVIDUALS NOT RECEIVING GOLD THERAPY

Sample	Concentration of tissue in assay samples mg/ml	Response		Equivalent gold content mcg/g	
		x1	x3	x1	x3
hair	3.98	3, 2.5, 2.5		0.499	
hair	11.9	1, 1, 1		0.230	
hair	44.6	5, 5, 4.5, 4.5[1]		0.088	
hair	0.605	0.5	1.8, 1.9	0	0.642
nail	0.655	0.5	1.5, 1.8	0	0.494
plasma	10.816	0.5, 0.2	1.3, 1.2	0	0.018[2]
skin	about 1	0, 0.5		0	

[1] noisy signal.

[2] mcg/ml.

(iii) NEUTRON ACTIVATION ANALYSIS OF GOLD

Non-destructive neutron activation analysis of gold in skin samples, or blank plasma with known added amount of gold sodium thiomalate was performed by commercial activation analysis services. Samples were weighed, freeze dried in plastic tubes, re-weighed, and then shipped for activation analysis. Conditions of analysis by Intelcom Rad Tech Activation Analysis Service, Box 80817, San Diego, California, CA 92138: 30 minutes irradiation in thermal neutron flux of 1.8 x 10^{12} n/cm^2-sec; count 0.411 MeV gamma of activated ^{198}Au. Conditions for analysis by Atomic Energy of Canada Limited Commercial Products Activation Analysis Service, Box 3100, Station C, Ottawa, K1Y 4J3: 330 minutes irradiation in a thermal neutron flux of 10^{12} n/cm^2-sec. Table 9 shows results of the analysis of plasma samples containing known amounts of gold. Table 10 shows a comparison of the results for aliquots of the same skin sample analyzed by both neutron activation analysis and flameless atomic absorption spectroscopy.

TABLE 9 NEUTRON ACTIVATION ANALYSIS OF GOLD ADDED TO BLANK PLASMA[1]

Volume of plasma ml	gold mcg Known	gold mcg Found[2]	% of true value
Intelcom Rad Tech			
0.010	0.04	0.041	102
0.025	0.10	0.100	100
0.050	0.20	0.190	95
Atomic Energy of Canada LIMITED			
0.025	0.10	0.100	100
0.050	0.20	0.190	95

[1] Plasma contained 4 mcg/ml gold as gold sodium thiomalate.

[2] $r=0.9997$, $p<0.001$, $df=3$, $Y = 0.9224\ X + 0.0061$, where Y = found, X = known gold content.

TABLE 10 CORRELATION[1] OF GOLD CONCENTRATION (MCG/G WET TISSUE) IN SKIN, ANALYSIS BY NEUTRON ACTIVATION ANALYSIS AND BY FLAMELESS ATOMIC ABSORPTION

NAA		AAS	
tissue weight mg	gold mcg/g	tissue weight mg	gold mcg/g
1.52	7.895	0.0235	10.284
3.28	11.890	0.0660	10.727
3.51	12.251	0.0685	15.010
1.47	25.850	0.0250	38.000
2.46	65.041	0.0535	70.992
0.96	100.000	0.0735	139.962

[1] $r=0.9882$, $p<0.001$ $df=4$, regression equation $Y = 0.7240\ X + 2.7682$, where X = concentration of gold by atomic absorption spectroscopy, Y = concentration of gold by neutron activation analysis.

The results of the two methods were well correlated; however atomic absorption results were higher than neutron activation analysis results. There was no correlation between weight of tissue analyzed and concentration of gold determined for the tissue for either method. This means that blank absorption of light by tissue does not account for the higher values obtained by the atomic absorption method. This was not an unexpected finding because deuterium background correction was used with the atomic absorption method, and also because blank values in one method would destroy the correlation of results by the two methods.

C. ANALYSIS OF CORTISOL IN PLASMA

(i) COMPETITIVE PROTEIN BINDING RADIOASSAY

Plasma cortisol determinations were made by a competitive protein binding (CPB) radioassay which had previously been developed in this laboratory (Jeffery, 1971). One alteration was made in the method. A 1 ml aliquot of the water phase obtained after carbon tetrachloride washing was used for assay directly without evaporation of the water. Cortisol standards were prepared in appropriate concentrations in aqueous solution just prior to assay. This modification allowed considerable saving in time since a 3 hour evaporation stage was eliminated. The method was found to be satisfactory. For convenience a summary of the method is given.

Removal of transcortin from the plasma samples was achieved by alcohol precipitation. Two tenths of a ml of plasma was diluted with 0.1 ml of water; then 1 ml of 95% ethanol was added, and the samples were mixed for 2 minutes. After centrifugation the supernatant was collected. Another ml of alcohol was added to the precipitate, and the mixing and centrifugation was repeated. The combined supernatants were evaporated at reduced pressure in a freeze drying apparatus.

To separate cortisol and corticosterone the residue from the alcohol supernatant was dissolved in 2 ml of water and partitioned twice against 20 ml of carbon tetrachloride by mechanical mixing for 2 minutes. The cortisol remains in the water, while the corticosterone and other steroids less polar than cortisol go into the carbon tetrachloride. The carbon tetrachloride was discarded. One ml of the water phase was taken for assay.

Standards were prepared in duplicate in the desired range (0-40 ng/ml). Standards and samples were processed in parallel from here. One ml of transcortin isotope solution (5% human plasma diluted with water plus ^{3}H-cortisol at an approximate concentration of 6 microcuries/100 ml) was added to each of the standards and samples. These were mixed and heated to 45°C for 5 minutes. After cooling in an ice bath for 15 minutes, 60 mg of Fuller's earth was added to each tube. The mixture was shaken for 2 minutes on a horizonatal agitator, cooled 10 minutes in an ice bath, and then centrifuged. The supernatant was collected and recentrifuged. One half ml of the supernatant was added to 10 ml (or 5 ml) of Bray's solution, and the samples were counted twice for sufficient time to give 1% counting error with 2 sigma statistics. A linear regression equation of reciprocal of activity of standard (milliseconds / disintegration) versus ng of cortisol in the standard was computed. The equation was used to determine the quantitiy of cortisol in the samples from the reciprocal of activity of the sample.

Recovery correction was done by including two control plasma samples with each assay. One control sample contained only endogenous cortisol while the second contained 10 ng/0.1 ml of added cortisol. These control plasma samples were prepared in a group and one of each was used with each cortisol assay. The control plasma samples were processed in parallel with the samples. From the difference between the amount of cortisol detected in the plasma only control, and the amount detected in the plasma plus cortisol control, the recovery could be determined. The amount of cortisol corrected for recovery was calculated by dividing the uncorrected weight of cortisol by the fractional recovery factor. The results were expressed in ng/0.1 ml which is numerically equivalent to mcg/100 ml of plasma.

Because all centrifugation must be done at once, only 24 samples, controls and standards could be done per day due to limitation in the capacity of the available centrifuge. This allowed the assay of 14 samples, 2 controls and 8 standards in a given assay run.

(ii) DRUG INTERFERENCE IN ASSAY OF CORTISOL

Interference in cortisol determination by drugs the patients were known to be taking was checked by assaying aqueous solutions of cortisol (10 ng/ml) and comparing the response (milliseconds/disintegration) for solutions of cortisol only and solutions of cortisol plus drugs (Table 11). Most drugs did not interfere (Table 12) when assayed directly without carbon tetrachloride water partitioning. As might be expected from its similar structure to cortisol, prednisone did interfere (Table 13). Regression analysis of data of Table 13 for samples partitioned against carbon tetrachloride and water after correction for recovery, yields the equation $Y = 97.4 X + 11.7$, where X = prednisone concentration in mcg/ml in sample assayed and Y = cortisol in ng. From the slope, 0.01 mcg/ml of prednisone, which is equivalent to 10 ng of prednisone, appears to be 0.974 ng of cortisol. That is the interference of prednisone is about 10%. Because prednisone elevates 'cortisol' levels detected, while it physiologically depresses cortisol levels in plasma, it is possible that no effect of prednisone would be detected in patient samples.

TABLE 11 DRUGS AND CONCENTRATIONS USED FOR INTERFERENCE STUDY

Drug	Dose mg	Concentration of Solutions Assayed, mcg/ml[1]	
Acetylsalicylic Acid	2000	0.2	2.0
Sodium Salicylate	2000 (as ASA)	2.0	20.0
Codeine Phosphate	60	0.05	0.5
D-Propoxyphene	65	0.065	0.65
Gold Sodium Thiomalate	50	0.2	2.0
Prednisone	20	0.02	0.2
Methylprednisolone Acetate	80	0.1	1.0
Betamethasone	0.5	0.03	0.3
Indomethacin	200	0.2	2.0
Phenylbutazone	100	0.1	1.0
Caffeine Citrate	600	1.0	10.0
Caffeine	300	0.5	5.0
Amitriptyline Hydrochloride	25	0.05	0.5
Phenobartial Sodium	120	0.2	2.0
Glutethimide	500	0.5	5.0
Diazepam	20	0.02	0.2
Chlordiazepoxide Hydrochloride	40	0.05	0.5
Primidone	125	0.125	1.25
Propantheline Bromide	15	0.03	0.3
Ferrous Sulfate	300	0.5	5.0
Amethopterine	10	0.01	0.1
Ethinyl Estradiol	0.1	0.01	0.1
Thyroid	30	0.06	0.6
Chlorpheniramine Maleate	16	0.02	0.2
Orphenadrine Citrate	100	0.1	1.0
Sodium Citrate		0.6	6.0
Calcium Chloride		10	

[1] Concentration used was derived from the maximum therapeutic doses recommended for the drug. Equivalent plasma concentration is 10x solution concentration because in assay procedure the plasma is diluted 1 in 10 during sample preparation.

TABLE 12 DRUGS NOT INTERFERING IN CPB RADIOASSAY OF CORTISOL

Drug	Milliseconds/disintegration [1]			F[2]
Drug Level	0	Lower	Upper	
Acetylsalicylic Acid	2.394	2.534	2.434	0.5852
	2.555	2.478	2.428	
Sodium Salicylate	2.394	2.459	2.439	0.2993
	2.555	2.453	2.408	
Codeine Phosphate	2.394	2.516	2.485	0.1769
	2.555	2.484	2.432	
D-Propoxyphene	2.601	2.582	2.599	0.9361
	2.606	2.578	2.522	
Gold Sodium Thiomalate	2.492	2.481	2.517	3.1255
	2.359	2.541	2.498	
	2.472	2.547	2.524	
	2.468	2.519	2.580	
Methylprednisolone Acetate	2.882	2.914	3.071	1.8100
	2.862	2.827	2.916	
Betamethasone	3.402	3.422	3.279	1.2827
	3.374	3.453	3.546	
	3.338	3.398	3.366	
	3.328	3.491	3.417	
Indomethacin	3.140	2.857	2.984	0.309
	3.106	2.937	3.019	
	2.739	2.720	2.630	
	2.605	2.630	2.783	
Phenylbutazone	2.525	2.618	2.570	0.0125
	2.639	2.525	2.523	
	2.279	2.309	2.330	
	2.318	2.367	2.345	
Caffeine Citrate	2.525	2.572	2.496	0.0174
	2.639	2.566	2.523	
	2.279	2.309	2.372	
	2.318	2.238	2.321	
Caffeine	2.525	2.554	2.588	0.0031
	2.639	2.624	2.531	
	2.279	2.297	2.253	
	2.318	2.314	2.390	
	2.739	2.693	2.658	

	2.605	2.603	2.680	
Amitriptyline	2.098	2.008	1.962	3.0300
Hydrochloride	2.075		2.051	
Phenobarbital	2.394	2.434	2.490	0.2958
Sodium	2.555	2.417	2.444	
Glutethimide	2.606	2.611	2.577	0.4912
	2.601	2.575	2.597	
Diazepam	2.098	2.266	2.059	2.3189
	2.227	2.120	1.776	
Chlordiazepoxide	2.098	2.056	2.065	1.211
Hydrochloride	2.227	2.093	2.122	
Primidone	2.098	2.070	2.064	1.7831
	2.227	2.232	1.773	
Propantheline	3.374	3.272	3.331	0.6074
Bromide	3.338	3.358	3.380	
Ferrous	2.492	2.284	2.586	0.2923
Sulfate	2.359	2.439	2.427	
	2.472	2.550	2.495	
	2.468	2.526	2.450	
Amethopterin	2.888	2.858	2.912	0.6000
	2.862	2.889	2.877	
Ethinyl	2.888	2.811	2.828	0.8100
Estradiol	2.862	2.903	2.824	
Thyroid	3.374	3.355	3.519	0.1773
	3.338	3.357	3.290	
Chlorpheniramine	2.606	2.646	2.593	1.215
Maleate	2.601	2.629	2.644	
Orphenadrine	2.098	2.175	2.114	0.4123
Citrate	2.227	2.057	2.084	
Sodium	2.525	2.543	2.507	0.2811
Citrate	2.639	2.560	2.561	
	4.704	3.449	4.405	
	4.579	3.890	4.840	
Calcium	0.548	0.773		t=0.4
Chloride	0.805	0.730		

[1] Milliseconds/disintegration cannot be compared from one drug to the next because of different amounts of radioactivity from one experiment to the next.

[2] Analysis of variance, simple random design, used to detect differences. For calcium chloride an unpaired 't' test was used (Steel, 1960). All F and t values are non-significant.

TABLE 13 DRUGS INTERFERING IN CPB RADIOASSAY OF CORTISOL

Drug	Milliseconds/disintegration			F
Drug Level	0	Lower	Upper	
Prednisone	3.045	3.274	3.924	89.38
	3.098^1	3.373^1	3.824	$p<0.005$
Prednisone after carbon tetrachloride water partitioning	1.802	1.790	2.054	19.48
	1.726^2	1.876^2	2.124	$p<0.025$

[1] [2] Groups of values with the same superscript are not statistically significantly different at 1 and 5 % level of significance respectively by Duncan's multiple range test (Steel, 1960).

D. DETERMINATION OF CORTISOL BINDING PROPERTIES OF PLASMA

(i) GENERAL METHODS

a. COMPETITIVE ADSORPTION

An aqueous solution of the plasma sample for which binding properties were to be determined was prepared, containing approximately 10000 dpm/ml of ^{3}H-cortisol, and was allowed to equilibrate at room temperature for at least one hour. Usually, unless indicated otherwise, the concentration of this plasma solution was 5%. Because of the high specific activity of ^{3}H-cortisol the added cortisol is small relative to the endogenous cortisol content of the plasma, thus after equilibration of ^{3}H-cortisol and endogenous cortisol the ratio of bound to unbound cortisol in the plasma will be essentially the same as for plasma without added radioactive cortisol. For example:

endogenous cortisol

= 10 mcg% = 100 ng/ml

specfic activity

= 45 Ci/mM = 2.76 dpm/ng

added ^{3}H-cortisol

= 10000 dpm/0.05 ml !

= 200000 dpm/ml

= $(2 \times 10^{5})/(2.76 \times 10^{5})$

= 0.725 ng/ml cortisol

Aqueous solutions of cortisol were prepared just prior to use. One ml of plasma solution and one ml of one of the aqueous standards were mixed. Routinely, eight different concentrations of cortisol were used to determine a binding curve. The cortisol concentrations used depended on the dilution of the plasma (Table 14).

TABLE 14 CONCENTRATION OF CORTISOL SOLUTION USED IN MEASUREMENT OF CORTISOL BINDING PROPERTIES OF PLASMA

Initial Concentration Plasma Solution %	Initial Concentrations of cortisol solution ng/ml
3-5	0, 4, 10, 15, 20, 30, 60, 120
2-2.5	0, 2, 4, 10, 15, 20, 30, 60
0.5-1.5	0, 1, 2, 4, 10, 15, 20, 30

It should be noted that because of dilution of plasma solution with one ml of cortisol solution, the actual concentration of plasma for which binding is determined is 2.5% when the initial plasma solution was 5% plasma. Unless indicated otherwise plasma concentration for competitive adsorption refers to this final dilution. From here on, one of two procedures was used: one referred to as the 45°C procedure, the other referred to as the 8°C procedure, the temperature referring to the temperature of equilibration of radioactive and nonradioactive cortisol.

45°C procedure: The samples were placed in a water bath at 45°C and mixed intermittently during a 5 minute equilibration period. The samples were then placed in an ice bath for a 15 minute cooling period. At the end of this time 60 mg of Fuller's earth was added to each sample and they were shaken on a horizontal agitator for 2 minutes in order to separate bound and free cortisol. The samples were returned to the ice bath for a further 10 minutes, then centrifuged at 8°C for 10 minutes at a speed of 2000 rpm. The supernatant was removed and re-centrifuged . One half ml of this supernatant, representing bound cortisol, was added to Bray's solution and counted by liquid scintillation at least two times for 10 minutes. An aliquot of the initial plasma solution was also counted to ascertain total radioactivity of the solution. Because of limitations in the capacity of the centrifuge only 3 binding curves could be done at one time. In an eight hour day, 6 binding curves could be done.

8°C procedure: The samples were placed in a controlled temperature room at 8°C and equilibrated overnight with continuous gentle mixing. From this point on the samples were handled in groups of 24. Sixty mg of Fuller's earth was added to each sample and they were shaken vigorously for 2 minutes on a horizontal agitator. The samples were immediately centrifuged at 8°C for 10 minutes at 2000 rpm. Supernatants were re-centrifuged and 0.5 ml was counted as described above. This procedure allowed for determination of 15 binding curves in a day and a half.

b. EQUILIBRIUM DIALYSIS

Because removal of unbound steroid by Fuller's earth was not complete, it was necessary to have a method with known efficiency for removal of unbound steroid in order to calculate the efficiency of the adsorbent. Equilibrium dialysis was used for this purpose. Plasma and cortisol solutions were prepared as described above except that dilutions were done with Ringer's solution. A measured volume of plasma solution was placed inside a pre-soaked dialysis bag prepared by knotting a strip of one inch dialysis tubing on itself. The dialysis bag was closed by knotting the other end on itself. Excess dialysis membrane was trimmed from the knots. The dialysis bags were then placed in 25 ml Erlenmeyer flask containing a measured volume of cortisol solutions of the desired concentration (Table 14). The samples were equilibrated at 8°C overnight while being continuously shaken gently. After equilibration, 0.5 ml of protein solution was removed from inside the dialysis bag using a syringe. One half ml of outer solution was also taken for counting as well as 0.5 ml of the initial plasma solution. This procedure was more tedious than the competitive adsorption procedure because of the preparation of the dialysis bags.

(ii) ADSORPTION CONSTANTS

The adsorption efficiency of Fuller's earth was determined at three plasma concentrations for several concentrations of added cortisol. Binding of cortisol in equivalent samples processed by equilibrium dialysis and by competitive adsorption was compared. For equivalent samples, the concentration of plasma in the diluted sample during competitive adsorption analysis, and inside the dialysis membrane for equilibrium dialysis, must be the same. Secondly, the concentration of cortisol in the diluted sample for competitive adsorption analysis must be equal to the total amount of cortisol in the equilibrium dialysis sample divided by the volume of solution inside the dialysis bag, that is the effective cortisol concentration inside the dialysis bag must equal the cortisol concentration in the final dilution in competitive adsorption analysis. If these two conditions are met, two samples are comparable. The Fuller's earth and the outer solvent in the dialysis experiment serve essentially the same purpose, which is the removal of unbound cortisol. Competitive adsorption was done by the 8°C procedure. Equilibrium dialysis was done using 2 ml of plasma solution inside the membrane and 2 ml of solution outside the membrane. The samples are described in Tables 15-17.

Adsorption constants were calcuated as shown below. Adsorption efficiency was standardized for volume of solution and weight of adsorbent.

Calculate U from equilibrium dialysis data

$U = (Te - P)/Ee$ (16)

correct B'c to same units of T as equilibrium dialysis data $B'cn = (Te \times B'ci)/Tc$ (17)

Calculate adsorption efficiency using B'cn from 17, U from 16 and Tc

$Ec = (Te - B'cn)/U$ (18)

Standardize adsorption efficiency for weight of adsorbent and volume of solution

$K = (Ec \times V)/M$ (19)

where U = true unbound concentration, dpm/0.5 ml

Te = dpm/0.5 ml of initial plasma solution used for equilibrium dialysis

Tc = dpm/0.5 ml of final dilution for competitive adsorption

P = apparent bound concentration by equilibrium dialysis = dpm/0.5 ml inside dialysis membrane

Ee = efficiency of removal of unbound cortisol by equilibrium dialysis = volume inside dialysis membrane/(total volume of dialysis system both inside and outside the membrane), specifically in this experiment = 2/(2+2)

B'ci = apparent bound concentration of cortisol by competitive adsorption as observed

B'cn = apparent bound concentration by competitive adsorption corrected to same activity as equilibrium dialysis data

K = adsorption constant in ml/g

M = weight of adsorbent in g

V = volume of solution to which adsorbent is added in ml.

The adsorption was constant over certain concentration ranges of added cortisol so average adsorption constants for these ranges were calculated and used in further work (Table 18).

TABLE 15 ADSORPTION EFFICIENCY OF FULLER'S EARTH FOR 2.5% PLASMA

Initial Conc of cortisol added, ng/ml		Cortisol Inside dialysis Membrane dpm/0.5 ml	Cortisol in Supernatant dpm/0.5 ml	Adsorption constant ml/g
ED[1]	CA[1]			
0	0	1747	769	30.4
2	4	1705	718	31.9
4	8	1562	675	24.7
5	10	1525	631	25.9
7.5	15	1430	569	25.2
10	20	1341	541	23.2
15	30	1263	507	22.3
20	40	1233	500	21.7
30	60	1194	480	21.5
60	120	1128	489	19.5

[1] ED equilibrium dialysis, CA competitive adsorption

[2] Ee = 2/(2+2) = 0.5 , Te = 2000, Tc = 1000

TABLE 16 ADSORPTION EFFICIENCY OF FULLER'S EARTH FOR 1.5% PLASMA

Initial Conc of cortisol added, ng/ml		Cortisol Inside dialysis Membrane dpm/0.5 ml	Cortisol in Supernatant dpm/0.5 ml	Adsorption constant ml/g
ED[1]	CA[1]			
2	4	1660	744	25.1
4	8	1647	680	30.2
5	10	1570	647	27.4
7.5	15	1360	596	21.0
10	20	1160	554	17.7
15	30	1162	527	18.8
20	40	1146	518	18.8
30	60	1091	520	17.6

[1] ED equilibrium dialysis, CA competitive adsorption

[2] Ee = 2/(2+2) = 0.5 , Te = 2000, Tc = 1000

TABLE 17 ADSORPTION EFFICENCY OF FULLER'S EARTH FOR 0.5% PLASMA

Initial Conc of cortisol added, ng/ml		Cortisol Inside dialysis Membrane dpm/0.5 ml	Cortisol in Supernatant dpm/0.5 ml	Adsorption constant ml/g
ED[1]	CA[1]			
0	0	1604	828	14.5
0.5	1	1572	661	26.4
1	2	1583	595	32.4
2	4	1491	524	31.2
4	8	1455	445	33.9
5	10	1390	407	32.4
7.5	15	1240	395	26.5
10	20	1098	399	22.2
15	30	1085	414	21.3

[1] ED equilibrium dialysis, CA competitive adsorption

[2] Ee = 2/(2+2) = 0.5 , Te = 2000, Tc = 1000

TABLE 18 ADSORPTION CONSTANTS FOR FULLER'S EARTH

% Plasma	Range of Added Cortisol ng/ml	Adsorption Constant ml/g mean ± standard deviation
2.5	0-4	31.1 ± 1.1
2.5	8-120	23.0 ± 2.2
1.5	0-10	27.6 ± 2.6
1.5	15-60	18.8 ± 1.4
0.5	0-30	26.8 ± 6.5

(iii) EXAMPLES OF DATA HANDLING FOR BINDING EXPERIMENTS

Raw count data from binding experiments were quench corrected as described (Methodology section A. (iii)). This data then must be transformed from count data into concentration of cortisol bound and unbound, prior to resolution by either method. For competitive adsorption experiments a program (Appendix I) was written which corrected for dilution of plasma, addition of cortisol as ^{3}H-cortisol, as endogenous cortisol and as added nonradioactive cortisol, adsorption efficiency of Fuller's earth and expressed the results as unbound and bound cortisol concentrations in ng/ml of experimental solution. The results were also expressed as bound/unbound and plotted as Scatchard plot of bound/unbound versus bound. The plot was necessary in order to choose points to use for computation by the limit method. A paper tape of the transformed data was also made. For equilibrium dialysis experiments another program was used to transform the data to yield output of the same format. Correction for dilution, added cortisol and efficiency of removal of unbound steroid were made. This transformed data was then entered into the resolving program (Appendix I). In the resolving programs corrections for dilution of plasma are made and parameters are expressed for undiluted plasma. Computer output from the three programs for a set of data for a competitive adsorption experiment and a similar set of data for an equilibrium dialysis experiment are included here. Explanatory notations have been given to clarify the input data required and the output of information obtained.

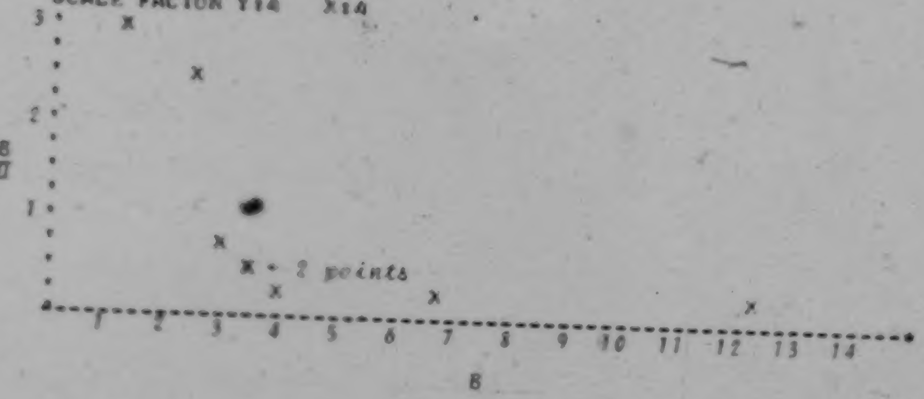
TRANSFORMATION OF PROTEIN BINDING DATA (5673A) - COMPETITIVE ADSORPTION

```
  GO

# REPLICATE COUNTS:1  Program will accommodate replicate counts.
SP ACT CI/MM:45
CBG %:5  Concentration of plasma in initial plasma dilution.
ENDO F UG%:6.11 Cortisol in plasma uncorrected for citrate dilution.
DPM/HALF ML CBG:1899 = 0.189900E+04 Activity of initial plasma dilution.
ADSORBENT G:0.06   ML:2

# DATA:8
ADSORP CONST   A NG/ML   DPM/HALF ML
:31.1 :0 :728
:31.1 :4 :694             Column 1 = adsorption constant.
:23.0 :10 :590
:23.0 :15 :541            Column 2 = concentration of cortisol solution
:23.0 :20 :496                       added to plasma solution.
:23.0 :30 :456
:23.0 :60 :435            Column 3 = activity of supernatant.
:23.0 :120 :426

 U        B   (NG/ML EXPTL SOLN)
   0.384    1.151
   1.019    2.515
   3.586    2.949         Subject:  85
   5.633    3.401         Sample:  24573
   7.984    3.550         8°C Procedure
  12.455    4.080
  24.764    6.770
  49.169   12.366
    B/U       B
   3.000    1.151
   2.467    2.515
   0.823    2.949
   0.604    3.401
   0.445    3.550
   0.328    4.080
   0.273    6.770
   0.252   12.366
SCALE FACTOR Y:4   X:4
```



LIMIT METHOD OF RESOLUTION OF PROTEIN BINDING DATA (5673A)

```
GO

INPUT DATA AS UNBOUND &BOUND UG/ML EXPTL SOLN (via paper tape)
                 N1 = number of data on initial straight line, Y=HX+G.
N1:4   N2:4   N2 = number of data on final straight line, Y=CX+D.

%CPC:2.0 (1) VOL:4.0 (2)
   U      B
:0.384 :1.151

:1.019 :2.515

:3.586 :2.042

:5.633 :3.401        Subject:  85
                     Sample:  24573
:7.984 :3.550        8°C Procedure

:12.455 :4.090

:24.764 :5.770

:49.169 :12.366

SD REGRESSION= 0.232575E+02 For Y=HX+G
SD REGRESSION= 0.249171E+01 For Y=CX+D
N1,UG%= 0.167379E+02
K1,L/M= 0.398724E+07
N2= 0.101927E+03
K2= 0.505036E+07.
```

(1) Percentage plasma in final dilution by competitive adsorption or percentage plasma inside dialysis membrane by equilibrium dialysis.

(2) Volume of original blood sample, for correction for citrate dilution.

```
        MATHEMATICAL RESOLUTION OF PROTEIN BINDING DATA (5673A)

*GO

N1:4   N2:4
```

N1 + N2 = number of data. Actual values of N1 and N2 are not important, since the sum of N1 and N2 are used in the program.

```
*CPG:2.5   VOL:4.6
   U        B
:0.394 :1.151

:1.019 :2.515

:3.596 :2.949

:5.633 :3.401

:7.994 :3.550

:10.455 :4.980

:24.764 :6.770

:49.169 :10.366

B1,NGM= 0.119616E+02 +- = 0.239906E+01  N= 0.210000E+02
K1,L/M= 0.137305E+10 +- = 0.361330E+10  N= 0.630000E+02
B2= 0.306171E+02 +- = 0.155636E+02  N= 0.200000E+01
K2= 0.499273E+09 +- = 0.520279E+08  N= 0.200000E+01*
```

Subject: 85
Sample: 24573
8°C Procedure

Mean ± standard deviation of parameter.
N = number of sets of data used to calculate the mean.

PROGRAM FOR TRANSFORMATION OF PROTEIN BINDING DATA - EQUILIBRIUM DIALYSIS

```
*C-FOCAL,69CF
*
*01.01 E
*01.04 A ! "N",N
*01.05 A ! "SP ACT CI/MM",SA;S SA=SA*.222E+13/.362E+9
*01.07 A !"CBG %",V;S V=V/100
*01.09 A !"ENDO F UG%",HC;S HC=HC*V/.1
*01.11 T !"DPM/HALF ML CBG";F I=1,N;A D;S SD=SD+D
*01.13 S D=SD/N;A !"VOL INNER SOLN =OUTER SOLN",VI
*01.15 A !"NUMBER OF DATA",M
*01.17 T !!"A NG/ML   DPM PROTEIN    DPM OUTSIDE"
*01.19 F I=1,M;D 2;S SP=0;S SO=0
*01.21 T !!"   U      B  (NG/ML INNER SOLN)"
*01.23 F I=1,M;X;D 3
*01.24 T "                            "
*01.25 F I=1,M;X;D 4
*01.26 T "                                         "
*01.36 A ! "SCALE FACTOR Y",Y,"  X",X
*01.40 F I=1,M;S BU(I)=FITR(BU(I)*Y+.5);S B(I)=FITR(B(I)*X+.5)
*01.42 S I=1;T !"'",#;D 6.05;D 6.06
*01.43 F I=2,M;D 6
*01.45 I (BU(M)-1)1.50;F J=1,BU(M);T !"'"
*01.50 T #;F JJ=1,60;T "-"
*01.64 Q
*
*02.01 A !A(I);F J=1,N;A P;S SP=SP+P
*02.02 F J=1,N;A O;S SO=SO+O
*02.05 S B(I)=SP/N;S U(I)=SO/N
*
*03.01 S T=(HC+2*D/SA)*VI+A(I)*VI
*03.02 S U=T*U(I)/(B(I)+U(I));S B(I)=T*B(I)/(B(I)+U(I))
*03.03 S B(I)=B(I)-U;S B(I)=B(I)/VI;S BU(I)=B(I)/U
*03.04 T !,% 8.03,U,B(I);X
*
*04.02 T !BU(I),B(I);X
*
*06.01 I (K-BU(I)-1)6.02;F J=1,K-BU(I);T !"'"
*06.02 T #
*06.05 F JJ=1,B(I);T " "
*06.06 T "X";S K=BU(I)
**
```

LIMIT METHOD OF RESOLUTION OF PROTEIN BINDING DATA (31773P)

```
GO

INPUT DATA AS UNBOUND &BOUND NG/ML EXPTL SOLN

N1:3   N2:5

%CBG:2.5    VOL:4.6
:  0.391 :   1.150

:    2.384 :   3.157

:    7.659 :   3.882

:   11.871 :   4.670

:   16.263 :   5.278

:   24.831 :   6.710

:   52.790 :   8.751

:  110.590 :  10.950
SD REGRESSION= 0.428616E+01
SD REGRESSION= 0.859630E+00
P1,UG%= 0.184028E+02
K1,L/M= 0.315937E+09
P2= 0.390577E+02
K2= 0.163985E+08*
```

Subject: 85
Sample: 24573

MATHEMATICAL RESOLUTION OF PROTEIN BINDING DATA (31773P)

```
 GO

N1:4   N2:4

%CPG:2.5   VOL:4.6
:    0.391 :    1.150

:    2.384 :    3.157

:    7.659 :    3.882

:   11.871 :    4.670

:   16.263 :    5.278

:   24.831 :    6.710

:   52.790 :    8.751

:  110.590 :   10.950

P1,UG%= 0.147806E+02 +- = 0.568401E+01  N= 0.290000E+02
K1,L/M= 0.308061E+09 +- = 0.408802E+09  N= 0.690000E+02
P2= 0.629296E+02 +- = 0.256467E+02  N= 0.230000E+02
K2= 0.650134E+07 +- = 0.415540E+07  N= 0.230000E+02*
```

Subject: 85
Sample: 24573

(iv) CALCULATION OF UNBOUND CORTISOL IN DILUTED PLASMA

Data from zero added cortisol on the cortisol binding curve was used to determine the proportion of endogenous plasma cortisol present in the protein bound and the unbound form. Dilution of plasma resulted in a lower proportion of bound cortisol than in undiluted plasma; however at the same dilution relative binding of cortisol in plasma could be determined. This gives an idea of the amount of active cortisol in plasma since unbound cortisol is usually considered to be active. Unbound cortisol was calculated as follows.

$U = (T - B \times 2)/(E \times T)$ as % unbound cortisol

$U = (T - B \times 2) \times F/(E \times T)$ as mcg% unbound cortisol

where

U = unbound cortisol expressed either as a percentage of total cortisol in plasma, or as a concentration of unbound cortisol in plasma.

T = dpm/0.5 ml of initial dilution of plasma used for determination of binding curve

B = dpm/0.5 ml of supernatant for competitive adsorption sample with no added cortisol. This must be multiplied times 2, as shown in formula, because of dilution during competitive adsoprtion procedure.

E = efficiency of removal of unbound cortisol by adsorbent which is adsorption constant (ml/g) x 0.06 g / 2 ml

F = endogenous cortisol concentration of plasma, corrected for citrate dilution.

(v) COMPARISON OF 8°C AND 45°C PROCEDURE

The 45°C and 8°C competitive adsorption procedures for determination of protein binding paratmeters were compared using plasma samples from three female control subjects (85,91,84). From a theoretical point of view, the 45°C procedure is inferior because the temperature at which the parameters are being determined is ambiguous. As expected binding capacities determined by the two procedures were the same (Table 19). However binding affinites and unbound cortisol levels, which should be affected by temperature differences, were also not significantly different. This suggests that in the 45°C procedure, conditions have equilibrated such that the temperature of the experiment is effectively the same as that for the 8°C procedure. Alternately the failure to find significant differences may be due to the large variability in the data, making real differences undetectable.

TABLE 19 EFFECT OF TEMPERATURE ON BINDING PROPERTIES[1]

Parameter	8°C	45°C	Difference[2] ± standard error
P1, mcg% (limit)	31.3	18.4	12.9 ± 12.7
P1, mcg% (math)	22.5	13.3	9.2 ± 6.5
K1, l/m x10^8 (limit)	3.68	3.70	0.01± 1.26
K1, l/m x10^8 (math)	1067.30	9.60	1057.70± 1059
P2, mcg% (limit)	92.1	37.2	54.8 ± 17.6
P2, mcg% (math)	313.8	54.6	259.2 ± 239.5
K2, l/m x10^7 (limit)	0.58	2.22	1.64± 0.72
K2, l/m x10^7 (math)	1.95	2.03	0.08± 2.05
Unbound, %	26.1	28.0	2.0 ± 1.2

[1] Subjects 85, 91, 84.

[2] All differences were non-significant by paired 't' test df=2.

(vi) EFFECT OF SOLVENT ON BINDING PROPERTIES

The literature indicates that binding experiments have been conducted using plasma diluted with isotonic solvent or plasma samples diluted with water. The importance of making dilutions with isotonic solvent was checked by comparing the binding properties obtained for three pair of binding curves determined by competitive adsorption, one curve of each pair prepared using Ringer's solution as diluent, the other curve prepared using distilled water as diluent. No differences were found between the binding properties for the two solvents (Table 20). The variablity of this data was much smaller than in the previous experiment. In future, all competitive adsorption experiments were done using distilled water as diluent. Equilibrium dialysis experiments required dilution with isotonic solvent to prevent osmotic flow.

TABLE 20 EFFECT OF SOLVENT ON BINDING PROPERTIES[1]

Parameter	Distilled Water	Ringer's Solution	Difference[2] ± standard error
P1, mcg% (limit)	16.0	14.9	1.0 ± 2.0
P1, mcg% (math)	10.6	14.4	3.9 ± 1.7
K1, l/m x10^8 (limit)	4.03	3.48	0.55± 0.73
K1, l/m x10^8 (math)	6.50	2.80	3.70± 3.02
Unbound, %	26.8	27.0	0.2 ± 1.4

[1] Subject 85; two pairs of data 8°C procedure, one pair of data 45°C procedure.

[2] All differences were non-significant by paired 't' test, df=2.

(vii) EFFECT OF STORAGE CONDITIONS ON BINDING PROPERTIES

Since it would be impossible to determine the binding properties of plasma as soon as it was withdrawn from the patient it was necessary to store the plasma. In order to check whether storage had an effect on binding properties a randomized block experiment was conducted, using samples (four) as blocks and storage conditions (four) as treatments. Binding curves were determined by competitive adsorption. The storage conditions used were fresh plasma, refrigerated plasma (5°C), frozen plasma (-10°C), frozen plasma (-10°C) which was stored for 2 to 3 weeks. No significant differences were found for the various storage conditions (Tables 21-25); therefore frozen plasma was used for future experiments. The observed differences for blocks was due to the fact that subject 76 was taking oral contraceptives which elevate transcortin binding capacity (Lipsett, 1972; Bulbrook, 1973; Gaunt, 1968).

TABLE 21 EFFECT OF STORAGE CONDITIONS ON TRANSCORTIN BINDING CAPACITY[1]

	Storage Conditions			
Sample[2]	Fresh	Refrigerated	Frozen	Frozen and Stored
1	19.9	17.1	14.8	
	(10.5)	(7.8)	(10.9)	
2	14.1	14.3	16.2	16.7
	(10.3)	(10.4)	(11.5)	(12.0)
3	20.0	13.7	17.0	16.8
	(13.4)	(10.8)	(12.0)	(10.8)
4	32.2	32.2	27.1	44.5
	(30.3)		(24.7)	(32.4)

F blocks	19.37 (69.63)	p<0.01
treatments	2.16 (1.23)	ns
Error Mean Square	15.41 (4.53)	

[1] P1 by limit method, () P1 by mathematical method, mcg%.

[2] Samples 1-3 subject 85, sample 4 subject 76; sample 1 8°C procedure, Samples 2-4 45°C procedure.

TABLE 22 EFFECT OF STORAGE CONDITIONS ON TRANSCORTIN BINDING AFFINITY[1]

Sample[2]	Storage Conditions			
	Fresh	Refrigerated	Frozen	Frozen and Stored
1	2.43	2.31	2.94	
	(3.62)	(4.25)	(5.44)	
2	4.44	3.96	3.87	3.99
	(19.26)	(10.06)	(4.43)	(13.73)
3	2.01	5.70	3.76	2.98
	(6.55)	(5.83)	(7.28)	(3.36)
4	6.07	2.53	4.03	3.72
	(4.56)	(13.06)	(2.89)	(5.80)

F blocks	1.39 (2.24)	ns
treatments	0.14 (0.54)	ns
Error Mean Square	1.71 (19.49)	

[1] K1 by limit method, () K1 by mathematical method, 1/m x10^8.

[2] Samples 1-3 subject 85, sample 4 subject 76; sample 1 8°C procedure, samples 2-4 45°C procedure.

TABLE 23 EFFECT OF STORAGE CONDITIONS ON ALBUMIN BINDING CAPACITY[1]

Sample[2]	Storage Conditions Fresh	Refrigerated	Frozen	Frozen and Stored
1		54.4	20.4	
	(20.5)	(152.4)	(148.8)	
2	18.0	31.2	51.1	101.8
	(35.1)	(328.4)	(95.2)	
3	71.6	40.3	44.6	35.0
	(47.6)	(394.9)	(32.1)	(269.5)
4	61.5	47.1	53.5	99.7
	(424.4)		(22.7)	(291.4)

F blocks	0.79 (1.35) ns
treatments	1.27 (2.35) ns
Error Mean Square	745.52 (20390.7)

[1] P2 by limit method, () P2 by mathematical method, mcg%.

[2] Samples 1-3 subject 85, sample 4 subject 76; sample 1 8°C procedure, samples 2-4 45°C procedure.

TABLE 24 EFFECT OF STORAGE CONDITIONS ON ALBUMIN BINDING AFFINITY[1]

		Storage Conditions		
Sample[2]	Fresh	Refrigerated	Frozen	Frozen and Stored
1		1.94	3.53	
	(9.53)	(0.83)	(0.65)	
2	4.64	2.17	1.49	0.60
	(1.44)	(2.47)	(0.43)	(4.98)
3	1.05	2.20	1.91	2.31
	(1.90)	(0.46)	(1.91)	(0.82)
4	2.03	1.50	1.29	2.02
	(0.32)		(1.27)	(1.04)

F blocks	0.20	(1.34)	ns
treatments	0.46	(0.97)	ns
Error Mean Square	1.60	(6.90)	

---

[1] K2 by limit method, () K2 by mathematical method, 1/m x10^7.

[2] Samples 1-3 subject 85, sample 4 subject 76; sample 1 8°C procedure, samples 2-4 45°C procedure.

TABLE 25 EFFECT OF STORAGE CONDITIONS ON UNBOUND CORTISOL, %

Sample[1]	Storage Conditions Fresh	Refrigerated	Frozen	Frozen and Stored
1	29.6	28.1	30.4	
2	25.9	27.8	25.7	24.6
3	31.9	23.0	24.9	28.4
4	25.5	28.7	37.2	9.5
F blocks	0.11 ns			
treatments	1.29 ns			
Error Mean Square	41.57			

[1] Samples 1-3 subject 85, sample 4 subject 76; sample 1 8°C procedure, samples 2-4 45°C procedure.

(viii) PROTEIN BINDING PROPERTIES FOR NORMAL INDIVIDUALS

Cortisol protein binding parameters of plasma from normal individuals were determined by competitive adsorption. As expected P1 was higher in estrogen treated females than in non-estrogen treated females or in males (Table 26). Comparison of these results to the results in Table 3 indicates that the values of P1 are similar to those obtained by other methods. The limit method gave larger estimates of P1 than did the mathematical method. K1 was similar to literature values when comparing determinations made at the same temperatures. The limit method gave lower estimates of K1 than did the mathematical method. P2 was low, although limited information was available from the literature for comparison, while K2 was high. P2 is highly variable as indicated by the large error mean square. Transcortin binding capacity was not saturated by endogenous cortisol in plasma as expected. Both unbound and total cortisol levels were increased in estrogen treated females compared to males despite an increase in the transcortin binding capacity of the plasma of these females.

The variation in binding properties of plasma taken from a normal individual at three to four month intervals was relatively small for most parameters (Table 27).

TABLE 26 CORTISOL BINDING PROPERTIES OF PLASMA FROM NORMAL INDIVIDUALS

Parameter[1]	Males	Females Estrogen treated	Females Not Estrogen treated	Overall Mean	Error[2] Mean Square
	n=8	n=3	n=8		
P1 (limit)	18.8	42.4	23.7	24.6	113.4[3]
P1 (math)	14.6	31.5	14.0	17.0	17.6[4]
K1 (limit)	4.76	2.84	3.25	3.82	3.27
K1 (math)	11.08	4.61	5.20	7.71	93.15
P2 (limit)	371.9	57.3	147.0	232.0	234811
P2 (math)	141.9	204.1	486.5	315.0	88076.7
K2 (limit)	0.53	2.35	0.73	0.91	0.241[5]
K2 (math)	1.65	1.20	0.75	1.15	1.24
Unbound, mcg%	2.0	5.2	3.2	3.0	2.87[6]
Unbound, %	23.7	21.7	26.3	24.5	35.23
Total cortisol mcg%	8.6	19.3	11.8	11.7	36.07[7]

[1] Units are the same as in Table 19.

[2] From analysis of variance, randomized design, treatments are males, females (estrogen), females (no estrogens). Least significant difference test to determine differences for individual means. Differences are non-significant unless indicated otherwise.

[3] $F=5.42$, $p<0.05$, $df=2/16$; $18.8<42.4$ $p<0.01$; $23.7<42.4$ $p<0.05$.

[4] $F=21.43$, $p<0.01$, $df=2/16$; $14.6<31.5$ $p<0.01$; $14.0<31.5$ $p<0.01$.

[5] $F=15.89$, $p<0.01$, $df=2/15$; $0.53<2.35$ $p<0.01$; $0.73<2.35$ $p<0.01$.

[6] $F=3.88$, $p<0.05$, $df=2/16$; $2.0<5.2$ $p<0.05$.

[7] $F=3.456$, $p<0.10$, ns, $df=2/16$; $8.6<19.3$ $p<0.05$)

TABLE 27 THE VARIATION IN CORTISOL BINDING PROPERTIES OF PLASMA FROM A SINGLE INDIVIDUAL[1]

Parameter[2]	Day 1	Day 2	Day 3	Overall Mean	Error Mean Square[3]
P1 (limit)	20.0	18.4	16.1	16.8	4.44
P1 (math)	16.5	13.4	11.5	12.2	1.43[4]
K1 (limit)	3.86	4.55	3.77	3.90	0.95
K1 (math)	4.87	5.70	8.20	7.50	25.27
P2 (limit)	83.7	57.8	52.9	56.2	731.13
P2 (math)		309.4	214.5	233.4	35842.9
K2 (limit)	0.59	2.56	1.90	1.84	1.46
K2 (math)	3.72	1.36	1.66	1.78	1.94
Unbound	29.7	19.6	26.8	25.8	9.39

[1] Subject 85; sampling days 9.11.72, 25.1.73, 24.5.73.

[2] Units are the same as in Table 19.

[3] From analysis of variance, randomized design, treatments are days. Least significant difference test to detrmine differences for individual means. Differences are non-significant unless indicated otherwise.

[4] $F=9.00$, $p<0.05$, $df=2/9$; $16.5>11.5$, $p<0.01$.

(ix) PRECISION OF EXPERIMENTALLY DETERMINED BINDING PARAMETERS

The precision of determination of cortisol binding properties of plasma by competitive adsorption was assessed by determination of binding curves for several plasma samples from subject 85. Both the 45°C and the 8°C procedures were used. Precision was expressed as standard deviation of estimated parameters for duplicate determinations (Snedecor, 1952) (Table 28). There was no clear cut advantage of one procedure over the other when comparing precision. The limit method gave more precise estimates of K1, P2, and K2 while the mathematical method gave more precise estimates of P1. The 8°C procedure was used in patient studies because it allowed handling of more samples in a given period of time. Both methods of resolving binding data were used in patient studies.

TABLE 28 STANDARD DEVIATION OF DUPLICATE DETERMINATIONS OF BINDING PARAMETERS OF PLASMA[1]

Parameter	45°C[2]	8°C
P1 (limit), mcg%	1.0 (5)	2.1 (4)
P1 (math), mcg%	0.8 (5)	1.2 (4)
K1 (limit), 1/m x10^8	0.57 (5)	1.12 (4)
K1 (math), 1/m x10^8	9.23 (5)	6.07 (4)
P2 (limit), mcg%	45.0 (5)	30.8 (4)
P2 (math), mcg%	2407.6 (3)	23998.7 (4)
K2 (limit), 1/m x10^7	0.84 (5)	1.41 (4)
K2 (math) 1/m x10^7	2.17 (3)	1.72 (4)
Unbound cortisol mcg%	0.48 (6)	0.21 (4)
Unbound cortisol, %	6.9 (6)	3.0 (4)

[1] Subject 85.

[2] Standard deviation of duplicates (number of duplicates).

STUDIES OF RHEUMATOID ARTHRITIS PATIENTS

A. DESCRIPTION OF STUDY POPULATION

The subjects for the study of gold effects on circulating cortisol consisted of three groups: I- patients receiving gold therapy, II- patients receiving other forms of therapy but not gold, III- control subjects with no disease. Characteristics of the three groups are given in Table 29. For greater details see Appendix II. For the purpose of classification, complicating illnesses were divided into those affecting endocrine organs, and other complicating illnesses, because it was felt the former were more likely to be of importance to the cortisol levels determined. Endocrine function can be affected by such things as the severity of illness, due to increased stress, but because of the difficulty of measuring this in patients with rheumatoid arthritis it was decided to ignore this factor. Patients were in attendance at a University of Alberta Hospital outpatient clinic set up for gold patients or were private patients of physicians of University of Alberta Hospital. Control subjects were healthy volunteers.

Blood samples (sodium citrate as anticoagulant) were taken between 12 noon and 3 pm. For gold patients samples were taken on the day of injection but prior to dosing thus plasma gold and plasma cortisol levels for these patients reflect previously administered gold. Blood samples were centrifuged and frozen in measured aliquots until assayed for gold or cortisol. Particular samples are identified by the week during the study in which they were taken; this time has no relation to the duration of gold therapy for a particular patient since patients entered the study at various stages of gold therapy. All gold was given intramuscularly as gold sodium thiomalate. The usual dosage schedule (expressed as mg of gold not as gold salt) was 5 mg first week, 12.5 mg second week, 25mg/week till 500 mg of gold had been given, then, if gold is definitely effective, 25mg/2 weeks x2, 25 mg/3 weeks x2, and finally 25 mg/month indefinitely. In this study, dose was expressed as either total dose, dosage schedule or dose rate. Total dose is the total accumulated dose the patient has received. Dosage schedule is the dose given and the time between dosing. Dose rate is the average mg/week of gold given regardless of the actual dosing schedule. As an example, suppose a patient were just starting gold theapy and received 5 mg the first week, 12.5 mg the second week, 25 mg the third week, 25 mg the fourth week, 25 mg the sixth week, 25 mg the ninth week, and 25 mg the thirteenth week. Because blood samples are taken prior to the administration of gold the dose exerting its effect on the body is the previous dose. The patients total dose would be 0 at week 1, 5 mg at week 2, 17.5 mg at week 3, 42.5 mg at week 4, 67.5 mg at week 6, 92.5 mg at week 9 and 117.5 mg at week 13. His dosage schedule would be 0 mg/week at week 1, 5 mg/week at week 2, 12.5 mg/week at week 3, 25 mg/week at week 4, 25mg/2 weeks at week 6, 25 mg/3 weeks at week 9, and 25 mg/4 weeks at week 13. His dose rate would be 0 mg/week at week 1, 5 mg/week at week 2, 12.5 mg/week at week 3, 25 mg/week at week 4, 12.5 mg/week at week 6, 8.33 mg/week at week 9, and 6.25 mg/week at week 13.

Patients were at different stages of gold therapy, including initiation to gold therapy, so all dose rates were represented. Multiple blood samples were obtained from many patients; some patients were followed for up to 23 weeks. Drugs the patients were receiving during the study period are shown in Table 30. This data was taken from the patients medical records.

TABLE 29 SUBJECT CHARACTERISTICS

Characteristic	Group I Gold	II No Gold	III Control
Number of subjects	50	24	21
Number of blood samples obtained	244	30	21
Sex	13 M 37 F (.26/.74)[1]	10 M 14 F (.42/.58)	9 M 12 F (.43/.57)
Age	6-72 (mean 48)	11-72 (mean 45)	19-39 (mean 25)
Diagnosis			
Rheumatoid Arthritis[2]	48 (0.96)	18 (0.75)	
Psoriatic Arthritis	2 (0.04)	4 (0.17)	
Dermatomyositis	0	1 (0.04)	
Osteoarthritis	0	1 (0.04)	
Complicating Illness			
Endocrine[3]	5 (0.10)	5 (0.21)	
Other[4]	19 (0.28)	7 (0.29)	
Both	4 (0.08)	4 (0.17)	
None	30 (0.60)	16 (0.67)	

[1] () fraction of patients in listed category.

[2] Classical or definite rheumatoid arthritis (Ropes, 1959).

[3] Thyroid disorders, ovarian disorders, drug-induced Cushingoid syndrome.

[4] Hypertension, heart disease, anxiety, depression, alcoholism, ulcers, osteoporosis, asthma, history of allergies, anemia, myositis-arteritis, peripheral neuropathy, hepatomegaly.

TABLE 30 DRUG THERAPY

Group	Drugs	Number of patients
I Gold	no other drugs	6
	salicylates only	36
	salicylates + other drugs[1]	8
II No Gold	no drugs	1
	salicylates only	10
	indomethacin only	2
	amethopterin only	2
	salicylates + other drugs[2]	7
	other drugs[3] (no salicylates)	2
III Control	no drugs	16
	estrogen/progesterone	3
	tetracycline	2

[1] Includes 3 patients on prednisone, 3 on intra-articular steroids, 1 on estrogens, 3 on propoxyphene, 1 on codeine, 1 on diazepam, 1 on phenobarbital, 1 on primidone, 1 on glutethimide, 2 on amitriptyline, 1 on caffeine, 1 on chlorpheniramine, 1 on ferrous sulfate, 3 on laxatives.

[2] Includes 2 patients on prednisone, 1 on betamethasone, 2 on intra-articular steroids, 1 on indomethacin, 1 on amethopterin, 1 on propoxyphene, 1 on chlordiazepoxide, 1 on orphenadrine, 1 on vitamin mineral supplements.

[3] Includes 1 patient on ACTH, 1 on propantheline bromide-phenobarbital, 1 on thyroid, 1 on estrogens, 2 on vitamin supplements.

The subjects for the study of the effect of gold on plasma cortisol binding were selected from the subjects described in Table 29. The four novice gold patients from group I formed group A. Here each patient served as his own control in a randomized complete block design experiment. Cortisol binding was measured in three plasma samples for each patient: prior to any gold, one week following first dose of gold, and when plasma gold level was greater than 2 mcg/ml (week 5 or later of gold therapy) (Table 31). Group B consisted of patients from group I paired, on the basis of age, sex, diagnosis, other drug therapy, and complicating illness, with patients from group II (Table 32). In group C patients from group I and II were grouped according to plasma gold levels into groups as shown in Table 33. Plasma samples to be analyzed for cortisol binding were selected randomly from all samples at that plasma gold level. This meant that in some cases a particular patient might be represented more than once. Cortisol binding parameters determined for all patients in group III (non-disease) have already been reported (Table 26). Unless indicated otherwise the plasma concentration used was 2.5% for the final dilution in competitive adsorption experiments.

TABLE 31 WEEK OF STUDY, PLASMA GOLD LEVEL (MCG/ML) (IN PARENTHESIS) AND TOTAL GOLD DOSE (MG) FOR NOVICE GOLD PATIENTS IN GROUP A OF CORTISOL BINDING STUDY

Patient	Week of Study		
	'Pre-gold'	'First gold dose'	'Plasma gold > 2 mcg/ml'
35	1	2 (0.46)	6 (3.38)
8	1	2 (0.75)	5 (2.95) [1]
25	1	2 (0)	14 (3.30)
26	1	2 (0)	5 (2.35)
Total gold dose	0	5	≥ 42.5

[1] Plasma concentration used was 0.5%.

TABLE 32 CHARACTERISTICS OF PATIENTS IN GROUP B OF CORTISOL BINDING STUDY

Patients	Week of Study	Age	Sex	Diagnosis	Complicating Illness	Drugs
2	2	63	M	RA		gold ASA[1] glutethimide propoxyphene
63	1	65	M	RA		ASA indomethacin
22[3]	1	50	F	RA		gold ASA
59	1	48	F	RA		ASA
30	1	61	F	RA		gold ASA
65	1	55	F	RA		ASA
31	13	52	M	RA	duodenal ulcer	gold ASA
60	1	51	M	RA		ASA
35[4]	8	52	F	RA	thyroidectomy history hypertension	gold ASA
14[3]	1	44		RA	History Goitre Anxiety Amenorrhea	ASA Propoxyphene Chlordiazepoxide Orphenadrine
36	13	45	F	RA		Gold ASA
70	1	46	F	RA	Biliary Cirrhosis	ASA
38	8,20	65		RA	sensitive to penicillin hiatus hernia	gold ASA ia[2] steroids
69	1	64	F	RA		indomethacin
42[3]	1	50	F	RA		gold ASA
61	1	52	F	RA		ASA
44	3	59	M	RA		gold ASA
64	1	53	M	RA		ASA

[1] acetylsalicylic acid
[2] intra-articular
[3] plasma concentration used 0.5%
[4] plasma concentration used 1.5%

TABLE 33 PLASMA GOLD LEVEL, AND WEEK OF STUDY FOR PATIENTS IN GROUP C FOR CORTISOL BINDING STUDY

Plasma Gold level mcg/ml	Patient (Week of Study)				
no gold	25 (1)	61 (1)	64 (1)	35 (1)	65 (1)
not detectable	30 (1)	36 (3)	38 (9)	25 (2)	40 (1)
<1	52 (2)	44 (1)	35 (2)	8 (2)	31 (13)
1-<2	44 (3)	46 (18)	38 (18)	7 (9)	
2-<3	54 (6)	25 (6)	46 (4)	26 (5)	22 (14)
3-<4	2 (2)	35 (6)	25 (14)	28 (16)	
4-<5	54 (7)	11 (17)			
5-<6	11 (8)	11 (9)	46 (10)	2 (4)	
>6	2 (5)	11 (22)			

The patients for the study on gold quantitation in skin were from group I or were not included in the previous study (Table 34). Patients not included in the previous study have letter codes rather than numeral codes. Four mm punch skin biopsies were taken from the patients' upper arm. Simultaneous blood samples (no anticoagulant or citrate), hair (distal to the scalp) and fingernail specimens were obtained from most of these patients.

TABLE 34 CHARACTERISTICS OF PATIENTS IN STUDY OF GOLD QUANTITIATION IN SKIN

Patient	Group	Age	Sex	Diagnosis	Dermatitis Associated with Gold Therapy
W[1]	Chrysiasis	61	F	RA	5 years ago
51	Chrysiasis	72	F	RA	7 years ago
33	Chrysiasis	38	F	RA	none
12	Gold	53	F	RA	4 years ago
3	Gold	52	F	RA	none
31	Gold	52	M	RA	none
23	Gold	39	M	RA	none
21	Gold	64	M	RA	none
N	Low Gold		F	RA	
J	Low Gold	61	F	RA	

[1] Patient had been receiving penicillamine therapy for RA.

B. RESULTS PLASMA CORTISOL LEVELS

Plasma samples from groups I, II and III were analyzed for cortisol by competitive protein binding radioassay. The data obtained can be examined in several ways in order to discern any effect of gold therapy on plasma cortisol levels. The first approach was to compare those patients who received gold (group I) with those who did not (group II). The patients who started gold therapy during the study can be used as their own controls to make a similar comparison of cortisol levels prior to gold and during gold therapy. The second approach is to stratify the data on plasma cortisol levels according to the amount of gold available to produce an effect. This can be done in several ways. Total dose of gold can be used as an expression of the amount of gold. This has merit because gold is slowly excreted from the body and probably accumulates in the body. Total dose, however ignores temporal factors which are very important in the distribution and excretion of a drug. Dosage schedule takes consideration of temporal factors; however the dose cannot be described by a single number and this makes manipulation of the data difficult. Dose rate takes into account both the amount of gold given and the time since dosing.

Plasma cortisol levels in group I (gold) and group II (no gold) were not significantly different from each other but were higher than group III (control) (Table 35).

TABLE 35 MEAN PLASMA CORTISOL LEVELS IN PATIENTS RECEIVING GOLD OR NO GOLD AND IN CONTROLS

Group	Level mcg%[1]
I Gold	19.4 ± 0.58 (n=244)[2]
II No Gold	16.7 ± 1.75 (n=30)[3]
III Control	12.1 ± 1.33 (n=21)[2] [3]

[1] Mean ± standard error of mean (se), n= number of samples analyzed.

[2] Significantly different by unpaired 't' test (Steel, 1960); p=0.05.

[3] Significantly different by unpaired 't' test, p<0.001.

Mean plasma cortisol levels were calculated for grouped data based on the characteristics in Table 29. No correlation was found between plasma cortisol level and sex or diagnosis. A weak correlation was found between plasma cortisol level and age (r=0.1094, p=0.039, n=260). Plasma cortisol levels were higher in group I patients with endocrine complications than in group I patients with other types of complicating illnesses (Table 36). Average plasma cortisol levels for each patient were used in a similar analysis of variance, so each patient would have the same weight in the experiment despite unequal replication. This analysis gave the same interpretation as the analysis using all individual values.

TABLE 36 EFFECT OF COMPLICATING ILLNESSES ON PLASMA CORTISOL IN GOLD TREATED PATIENTS WITH VARIOUS COMPLICATING ILLNESSES

Group	Plasma Cortisol[1] mcg%	Sex	Age
No complicating Illness	19.0 (141)	8M 22F (0.27/0.73)	44 (6-64)
Endocrine Illness	21.8[3] (24)	0M 5F	51 (44-55)
No Endocrine but other Complicating Illness	16.6[3] (78)	5M 10F (0.33/0.67)	54 (31-72)

[1] Mean (number of samples analyzed). Significance by analysis of variance, random design with unequal replication (Steel, 1960) F=3.59, p<0.05, error df=240, error mean square=77.34.

[2] Proportion of each sex.

[3] Significantly different by least significant difference (lsd) for unequal replication (Steel, 1960), p<0.05.

Because of the few examples of most drugs, the effect of drugs on plasma cortisol levels could be determined only for salicylates. Comparisons were made among individual cortisol values for those subjects in group I receiving gold therapy and no other drugs, subjects in group I receiving salicylates and gold but no other drugs, subjects in group II receiving salicylates only, and subjects in group III receiving no drugs. The gold only group had significantly higher plasma cortisol than any other group. The gold +

salicylate subjects had higher cortisol levels than the control subjects, while the salicylate only and control subjects could not be distinguished from each other (Table 37). The gold and gold + salicylate groups each had two patients with endocrine disorders which may have contributed to the elevated cortisol levels, so these patients were excluded and the data re-examined (Table 38). This reduced the cortisol level for the gold only group. Now, gold and gold + salicylate were not significantly different from each other but were higher than the control group. The salicylate only group was not significantly different from the gold only or the gold + salicylate group or from the control. When average cortisol values were used in a similar analysis, excluding patients with endocrine disorders, the intrepretation was essentially the same. By matching each of the gold only patients, not suffering from any endocrine disorder, with a gold + salicylate only and also a salicylate only patient of similar age, sex, and diagnosis, these factors could be eliminated and the drug effect examined. Gold therapy in groups receiving gold was also matched to a similar total gold dose. The cortisol levels were not different for these three groups (Table 39).

TABLE 37 EFFECT OF GOLD AND SALICYLATE THERAPY ON PLASMA CORTISOL

Group	Plasma Cortisol[1] mcg%	Sex	Age
Gold only	24.5[2] (21)	1M 5F (0.17/0.83)	40.2 (18-55)
Gold + Salicylate	18.6[3] (175)	10M 26F (0.28/0.72)	48.5 (6-72)
Salicylate only	14.1 (10)	6M 4F (0.6/0.4)	37.6 (11-55)
Control no drugs	11.2[3] (15)	9M 6F (0.6/0.4)	27.1 (19-39)

[1] Mean (number of samples analyzed). By analysis of variance, random design with unequal replication, $F=6.91$, $p<0.005$, error df=217, error mean square=84.0.

[2] Significantly different from all other values by lsd, $p<0.01$.

[3] Significantly different by lsd, $p<0.01$.

TABLE 38 EFFECT OF GOLD AND SALICYLATE THERAPY ON PLASMA CORTISOL EXCLUDING PATIENTS WITH ENDOCRINE DISORDERS

Group	Plasma Cortisol[1] mcg%	Sex	Age
Gold only	18.9[2] (14)	1M 3F (0.25/0.75)	33.6 (18-46)
Gold + Salicylate	18.6[3] (163)	10M 24F (0.29/0.71)	48.5 (6-72)
Saliclyate only	14.1 (10)	6M 4F (0.6/0.4)	37.6 (11-55)
Control no drugs	11.2[2] [3] (15)	9M 6 F (0.6/0.4)	27.1 (19-39)

[1] Mean (number of samples). By analysis of variance, random design with unequal replication, $F=3.746$, $p<0.025$, error df=198, error mean square=81.3.

[2] Significantly different by lsd, $p<0.05$.

[3] Significantly different by lsd, $p<0.05$.

TABLE 39 EFFECT OF GOLD AND SALICYLATE THERAPY IN MATCHED PATIENTS

Group	Cortisol[1]	Patients
Gold only	18.6 ± 3.7	16 28 45 56
Gold + Salicylate	24.1 ± 2.5	26 29 46 40
Salicylate only	20.8 ± 5.2	26 25 70 65

[1] Mean ± se. Not significantly different by paired 't' test (Steel, 1960).

Table 40 is a tablulation of plasma cortisol levels in patients receiving steroid therapy during the study. Insufficient data is available for statistical analysis. In many cases there was a time lapse after steroid therapy before the next sample was taken. The values of plasma cortisol for patients on continuous steroid therapy were not grossly different from the overall cortisol levels for patients in the study as a whole.

TABLE 40 PLASMA CORTISOL LEVELS IN PATIENTS RECEIVING STEROID THERAPY

Patient	Plasma Cortisol mcg%	Steroid Therapy
Group I gold		
24	25.0 15.9 28.6	prednisone 10-15 mg/d
66	9.4	betamethasone 0.5 mg/d
67	12.8	prednisone 5mg/d
Group II no gold		
7	21.3 17.2 19.7 18.8[1] 23.3 19.6 22.8	prednisone 5mg on alternate days, [1] intra-articular steroid injection after this sample, 2 weeks until next sample.
46	12.7 12.3[1] 18.8 14.7 24.0 18.7 20.2 22.7 24.7 17.5	[1] oral steroids given after this sample 1 week lapse before next sample.
38	10.8 27.9 8.4[1] 9.7 30.5 14.2 7.4 14.2 13.4 9.9	[1] methylprednisolone acetate intra-articular after this samples, 1 month until next sample.

Four patients were initiated to gold therapy during the study period. It is possible to compare the cortisol levels in these patients prior to gold and during gold therapy (Table 41). Analysis of variance as a randomized complete block design (Steel, 1960) with patients as blocks and total gold dose as treatment for total doses up to 67.5 mg, revealed no significant differences.

TABLE 41 PLASMA CORTISOL LEVELS, MCG%, IN NOVICE GOLD PATIENTS

Patient	Total Dose of Gold, mg				
	0	5	17.5	42.5	67.5
8	25.6	31.2	29.3	35.8	22.6
25	19.2	25.3		22.1	23.2
26	39.4	35.5	31.2	24.0	18.2
35	21.3	14.4			25.4

F blocks	2.00 ns
treatments	0.58 ns
Error mean square	69.41

Cortisol data was also analyzed according to the amount of gold received. The gold dose can be expressed in several ways: total gold dose, dosage schedule, or dose rate. No correlation was found between plasma cortisol level and total gold dose for all patients in group I, for males only or for females only. If the data was split into two groups, less than 500 mg and greater than 500 mg total gold dose, there was still no correlation between plasma cortisol and total gold dose. A correlation was found between gold dose rate and plasma cortisol level in male patients ($r=0.3776$, $p=0.025$, $n=51$), but not in females. Dosage schedule cannot be used with correlation. The cortisol levels were grouped according to the dosage schedule and a Student-Neuman-Keuls comparison of ordered means (Steel, 1960) was performed to detect significant differences. Differences were detected for males (Table 42) but not for females. Dosage schedules used for grouping were 25mg/wk, 25 mg/2 wk, 25 mg/3 wk, 25 mg/4 wk, 12.5 mg/wk, 12.5 mg/2 wk, 12.5 mg/3 wk, 12.5 mg/4 wk and 5 mg/wk. In males, plasma cortisol levels were significantly higher when the patients were receiving 25 mg/wk than when the patients were receiving 12.5 mg/wk.

TABLE 42 EFFECT OF DOSAGE SCHEDULE ON PLASMA CORTISOL IN MALE PATIENTS

Dosage Schedule		25 mg/wk	25 mg/4 wk	12.5 mg/wk
Plasma Cortisol mcg%	mean	22.9[1]	15.9	10.0[1]
	se	3.8	1.4	0.9
	n	12	20	3

[1] Significantly different by Student-Neuman-Keuls comparison of ordered means, p<0.05.

The correlation coefficient for plasma cortisol level versus plasma gold level was calculated for the overall data, for individual patients (where 3 or more samples were obtained from that patient) and for the data grouped in various ways (by sex, age, diagnosis, complicating illness). A weak but significant correlation between plasma cortisol level and plasma gold level was found in male patients ($r=0.3313$, $p=0.006$, $n=57$). Other correlations were not significant. Analysis of variance, using a simple random design, with data grouped by plasma gold levels (Tables 43 and 44) also revealed that plasma cortisol levels were higher at high plasma gold levels (>4 mcg/ml) in male patients only. Use of average cortisol levels for patients appearing more than once at a given gold level gives more equal weight to each patient thus reducing the influence of an individual patient on the results, but not completely equalizing weighting for all patients because all patients do not appear at all plasma gold levels. Closer examination of the data revealed that patient 2 unduly weighs the values for high plasma gold levels. Handling the data such that patients appear as blocks and plasma gold level as treatments, requires selection of data only for patients who appear in several groups. Missing data can be handled by this experimental design. In females no relationship of plasma gold to plasma cortisol was found (Table 46), similar to the finding determined above by correlation. In males, a significant F indicates a difference in plasma cortisol for different plasma gold (Table 45). Only a few gold groups could be used for this analysis because of limited data. Missing values in analysis of variance complete block design, bias upward the treatment mean square (Steel, 1960) but do not bias the error mean square. This means that nonsignificant F values indicate lack of treatment effects fairly; however significant F values may be biased upward due to missing values, and therefore may not indicate treatment effects. This appears to be the case with the analysis for males since when the group with plasma gold levels of 2-<3 mcg/ml is eliminated, which reduces the number of missing values significantly, F is no longer signficant (Table 45). It can be concluded that the finding of a correlation between plasma cortisol and plasma gold in male patients is due to the over representation of a single patient (patient 2), and further that gold does not affect plasma cortisol levels in patients studied.

TABLE 43 PLASMA CORTISOL LEVELS IN MALE PATIENTS AT VARIOUS PLASMA GOLD LEVELS
ANALYSIS OF VARIANCE RANDOM DESIGN

Plasma Gold mcg/ml	Plasma Cortisol Level mcg%					Treatment means
no gold	20.8[1] (1) n=4	12.2 (17) n=1	39.4 (26) n=1	17.6 (57) n=1	3.7 (58) n=1	15.3[2] [3]
	20.2 (60) n=1	8.6 (63) n=1	14.6 (64) n=1	12.8 (67) n=1	2.8 (68) n=1	
not detectable	26.0 (15) n=1	14.7 (16) n=1	13.2 (20) n=3	11.7 (21) n=2	13.6 (23) n=3	18.9
	33.4 (26) n=2	13.7 (31) n=1	10.0 (39) n=1	33.6 (50) n=1	18.7 (52) n=1	
<1	14.5 (20) n=1	13.8 (21) n=2	20.8 (23) n=3	11.4 (31) n=3	16.6 (39) n=4	14.0[4]
	10.8 (44) n=1	10.4 (52) n=1				
1-<2	10.6 (15) n=1	23.4 (20) n=1	18.2 (26) n=1	8.7 (31) n=1	31.1 (39) n=1	18.5
	23.8 (44) n=1	13.5 (52) n=1				
2-<3	22.7 (26) n=1	11.0 (52) n=2				16.8
3-<4	23.0 (2) n=1	15.8 (19) n=1	30.6 (52) n=2			23.1
4-<5	36.0 (2) n=2					36.0[2]

5-<6	30.3 (2) n=1		30.3
>6	33.8 (2) n=2		33.8[3] [4]
F		1.736 ns	
Error Mean Square		71.55	

[1] Plasma cortisol level, () patient, n=number of samples.

[2] Significantly different by lsd, t=2.34, $p<0.05$, se=8.87.

[3] Significantly different by lsd, t=2.09, $p<0.05$, se=8.87.

[4] Significantly different by lsd, t=2.18, $p<0.05$, se=9.04.

TABLE 44 PLASMA CORTISOL LEVELS IN FEMALE PATIENTS AT VARIOUS PLASMA GOLD LEVELS
ANALYSIS OF VARIANCE RANDOM DESIGN

Plasma Gold mcg/ml	Plasma Cortisol Level mcg%					Treatment means
no gold	10.0[1] (6) n=2	25.6 (8) n=1	15.9 (10) n=1	26.5 (14) n1=	23.2 (25) n=3	16.1
	19.2 (25) n=1	31.2 (35) n=1	2.2 (59) n=1	1.6 (61) n=1	16.0 (62) n=1	
	7.7 (65) n=1	9.4 (66) n=1	13.7 (69) n=1	23.2 (70) n=1		
not detectable	15.3 (3) n=3	15.5 (4) n=1	20.3 (12) n=1	17.3 (13) n=1	25.3 (25) n=1	19.2
	8.4 (27) n=1	24.2 (30) n=1	25.2 (33) n=1	15.8 (36) n=3	21.4 (40) n=1	
	9.0 (41) n=1	12.8 (43) n=2	35.2 (45) n=1	20.8 (51) n=1	11.4 (53) n=3	
	27.3 (55) n=1	21.8 (56) n=1				
<1	23.2 (3) n=2	21.1 (4) n=1	11.7 (5) n=1	30.2 (8) n=2	18.3 (13) n=4	20.0
	23.0 (18) n=3	6.9 (22) n=1	23.3 (27) n=1	35.2 (32) n=1	22.0 (33) n=1	
	14.4 (35) n=1	17.8 (36) n=3	17.8 (37) n=2	15.9 (41) n=2	26.6 (42) n=3	
	10.8 (43) n=2	7.0 (48) n=1	20.8 (49) n=1	33.0 (51) n=5	21.5 (55) n=1	

<2	14.4 (5) n=1	20.8 (7) n=7	5.7 (11) n=1	17.5 (18) n=1	17.8 (22) n=2	17.9
	31.6 (25) n=2	18.8 (28) n=2	31.6 (29) n=6	20.1 (33) n=1	28.5 (34) n=2	
	25.1 (36) n=1	14.9 (37) n=1	11.9 (38) n=3	5.7 (41) n=1	21.1 (46) n=2	
	14.0 (47) n=3	7.2 (48) n=1	13.6 (49) n=2	22.6 (51) n=1	17.0 (53) n=3	
	25.2 (54) n=1	11.1 (55) n=3	15.8 (56) n=2			
2-<3	16.4 (3) n=1	7.8 (5) n=2	17.2 (7) n=1	29.2 (8) n=1	20.9 (22) n=2	18.4
	31.5 (25) n=3	18.6 (28) n=4	28.1 (29) n=3	24.8 (34) n=4	11.6 (38) n=2	
	12.5 (46) n=2	13.2 (47) n=4	7.0 (54) n=1			
3-<4	6.6 (5) n=2	45.0 (9) n=2	13.6 (11) n=2	27.6 (25) n=2	10.1 (28) n=1	19.2
	19.5 (35) n=3	18.8 (46) n=1	20.0 (47) n=4	11.5 (54) n=1		
4<5	11.1 (5) n=1	39.0 (9) n=1	13.0 (11) n=2	22.1 (35) n=2	17.9 (46) n=3	20.1
	34.0 (47) n=1	3.9 (54) n=1				
5-<6	15.3 (11) n=7	22.2 (35) n=2	23.4 (46) n=2	18.8 (47) n=1	14.7 (54) n=1	18.9

>6	11.4 (5) n=1	16.6 (11) n=2	7.5 (35) n=1	16.4 (54) n=1	13.0

F 0.522 ns
Error Mean Square 69.53

[1] Plasma cortisol level, () patient, n=number of samples.

TABLE 45 PLASMA CORTISOL LEVELS, MCG%, IN MALE PATIENTS AT VARIOUS PLASMA GOLD LEVELS
ANALYSIS OF VARIANCE RANDOMIZED COMPLETE BLOCK DESIGN

Patient	Plasma Gold Level, mcg/ml			
	not detectable	<1	1-<2	2-<3
15	26.0		10.6	
20	13.2	14.5	23.4	
21	11.7	13.8		
23	13.6	20.8		
26	33.4		18.2	22.7
31	13.7	11.4	8.7	
39	10.0	16.6	31.1	
44		10.8	23.8	
52	18.7	10.4	13.5	11.0
treatment means	17.7	14.0	18.5	16.8
F blocks	1.35 ns		(0.72)[1] ns	
treatments	4.90 $p<0.025$		(0.45) ns	
Error Mean Square	76.21		(61.97)	

[1] () Analysis of variance where group with gold level 2-<3 excluded.

TABLE 46 PLASMA CORTISOL LEVELS, MCG%, IN FEMALE PATIENTS AT VARIOUS PLASMA GOLD LEVELS
ANALYSIS OF VARIANCE RANDOMIZED COMPLETE BLOCK DESIGN

Patient			Plasma Gold Level mcg/ml						
	no gold	not detectable	<1	1-<2	2-<3	3-<4	4-<5	5-<6	>6
3		15.3	23.2		16.4				
5			11.7	14.4	7.8	6.6	11.1		11.4
8	25.6		30.2		29.2				
11				5.7		13.6	13.0	15.3	16.6
22			6.9	17.8	20.9				
25	19.2	25.3		31.6	31.5	27.6			
28				18.8	18.6	10.1			
33		25.2	22.0	20.1					
35	31.2		14.4			19.5	22.1	22.2	7.5
		15.8	17.8	25.1					
41		9.0	15.9	5.7					
46				21.1	12.5	18.8	17.9	23.4	
47				14.0	13.2	20.0	34.0	18.8	
54				25.2	7.0	11.5	3.9	14.7	16.4
55		27.3	21.5	11.1					
treatment means	25.3	19.6	18.2	17.6	17.5	16.0	17.0	18.9	13.5

F blocks	1.55 ns
treatments	2.00 ns
Error Mean Square	140.38

Side effects occurred in 63% of the gold patients at some time during therapy. Almost all reactions were mild. Further details of the adverse reactions are given in Table 47. Data for patients who experienced side effects were grouped according to samples taken before the reaction, samples taken while the patient was experiencing the reaction, and samples taken after the reaction had subsided. Patients who were free of side effects had higher cortisol levels than did patients after adverse reactions had subsided (Table 48). Patients had higher cortisol levels prior to side effects than after side effects had subsided; however with selection of patients with replicated samples before, during and after side effects the effect was no longer significant (TABLE 49).

TABLE 47 CLASSIFICATION OF SIDE EFFECTS IN GOLD PATIENTS

Type of Reaction	Time of Reaction	Number of Patients[1]
Severe Skin reaction	during study period	1
Mild skin reaction	prior to study period	13
Mild skin reaction	during study period	6
Mild skin reaction	up to 5 months after study period	4
Mild mucosal reaction	prior to study period	2
Mild mucosal reaction	during study period	3
Mild mucosal reaction	up to 5 months after study period	4
Proteinuria	prior to study period	7
Proteinuria	during study period	2

[1] Some patients exhibited more than one type of reaction on more than one occasion.

TABLE 48 EFFECT OF SIDE EFFECTS TO GOLD ON PLASMA CORTISOL LEVELS

Group	Plasma Cortisol[1] mean, mcg%	Number of Samples
No side effects	19.9[2]	87
Sample taken prior to side effects	20.3[3]	38
Samples taken during side effects	16.1	13
Samples taken after side effects subsided	16.9[2] [3]	99

[1] By analysis of variance, random design with unequal replication, F=2.68, $p<0.05$, error df=233, error mean square=78.44

[2] Significantly different by lsd, $p<0.05$.

[3] Significantly different by lsd, $p<0.05$.

TABLE 49 PLASMA CORTISOL LEVELS IN PATIENTS WITH SIDE EFFECTS TO GOLD
ANALYSIS OF VARIANCE RANDOMIZED COMPLETE BLOCK DESIGN

Patient	Time of Sample Relative to Side Effects Before	 During	 After
5	8.9	11.2	8.8
7	18.8	23.3	21.2
22		15.7	16.7
26	33.4		25.8
47	17.2	17.6	14.6
48	7.0		7.2
49	18.6	10.6	
53	3.7		15.7
Treatment means	15.4	15.7	16.1

F blocks	7.36 $p<0.005$
treatments	0.64 ns
Error Mean Square	23.43

C. RESULTS PLASMA GOLD LEVELS

Plasma gold levels for plasma from patients in group I were analyzed by flame atomic absorption spectroscopy. The effect of gold dose on plasma gold level was evaluated by expressing the gold dose as total dose, gold dose rate, and as dosage schedule. There was a positive correlation ($r=0.4368$, $p<0.001$, $n=134$) between plasma gold dose and plasma gold for total dose up to 500 mg. Up to 500 mg dosing is usually on a weekly basis. For total doses greater than 500 mg, the correlation with plasma gold level was negative ($r=-0.2406$, $p<0.02$, $n=106$). Gold dose rate and plasma gold level gave a strong correlation ($r=0.8366$, p=0.001, n=244, Y = 0.17 X - 0.23, where Y=plasma gold level mcg/ml, X=dose rate mg/wk). Plasma gold levels for different dosage schedules are shown in Table 50. The plasma gold levels were measured at the end of the dose interval so represent residual plasma gold levels. A dosage schedule of 25 mg/week produced the highest residual gold level; 25 mg/2 or 3 weeks and 12.5 mg/1 or 2 weeks produced approximately half the residual gold level but were indistinguishable from each other. The plasma gold level versus gold dose (expressed as mg, with no consideration of time) gave a correlation of 0.3336 (p=0.001, n=250) which is weaker than the correlation for plasma gold versus total dose (up to 500 mg) or gold dose rate. It can be seen that dose rate is most closely related to plasma gold level; therefore it has been used further to adjust gold levels to a common dose rate to aid in comparison of plasma gold levels.

TABLE 50 EFFECT OF GOLD DOSAGE SCHEDULE ON PLASMA GOLD LEVELS

Dosage Schedule	Plasma Gold Level[1] mcg/ml
25 mg/wk	4.21 ± 0.48 (n=62)
25 mg/2 wk	2.12 ± 0.09 (n=20)[2]
25 mg/3 wk	1.87 ± 0.19 (n=3)[2,3]
12.5 mg/2 wk	1.52 ± 0.23 (n=10)[2,3,4]
12.5 mg/wk	1.50 ± 0.20 (n=15)[2,3,4]
25 mg/4 wk	0.69 ± 0.08 (n=48)[3,4]
12.5 mg/4 wk	0.43 ± 0.11 (n=5)[4]
12.5 mg/3 wk	0.42 ± 0.20 (n=5)[3,4]
5 mg/wk	0.24 ± 0.16 (n=5)[3,4]

[1] Mean ± standard error of the mean (number of observations).

[2-4] Values with the same superscript are not significantly different by Student-Neuman-Keul multiple range test, $p<0.05$, standard error of difference (sed) =0.35.

Plasma gold levels were higher in females (2.00 mcg/ml, n=192) than in males (1.40 mcg/ml, n=51) while dose rate in females was only insignificantly higher than in males (females 12.5 mg/wk, males 11.8 mg/wk, Table 51). The use of analysis of covariance (Steel, 1960) allows elimination of differences due to varying dose rate, thus permitting examination of sex differences in plasma gold levels. The plasma gold levels were adjusted by regression to the plasma gold level which would be expected if all patients had received the overall mean dose rate of gold for that experiment.

TABLE 51 DIFFERENCE IN PLASMA GOLD LEVELS IN MALE AND FEMALE PATIENTS

Sex	Number of Samples	Mean Observed Plasma Gold mcg/ml	Mean Dose Rate mg/wk	Mean Adjusted Plasma Gold mcg/ml
Female	192	2.00	12.5	1.97
Male	51	1.40	11.8	1.51
Null hypothesis:no difference between groups[2]				
F		4.673 $p<0.05$ df 1/241	0.336 ns 1/241	9.457 $p<0.005$ 1/240

[1] Plasma gold level is adjusted by covariance to 12.3 mg/wk dose rate.

[2] Null hypothesis is rejected if level of significance, $p \leq 0.05$ for F. When null hypothesis is upheld ns, not signficant, is indicated with F. Df=degrees of freedom.

There was no consistent relationship between plasma gold level and age. The presence of complicating illness had no effect on plasma gold when it had been adjusted to constant dose rate by covariance (Table 52).

Those patients receiving gold and salicylates only and patients receiving gold only did not have significantly different plasma gold levels either before or after adjustment to a constant dose rate (Table 53).

Patients who developed side effects to gold did not handle gold any differently than those who did not, when comparing plasma gold levels adjusted by covariance to a constant dose rate (Table 54). Incidence of side effects to gold was not related to sex as judged by Fisher exact probability test (Siegel, 1956): 21 of 37 females and 10 of 13 males had side effects to gold. This will be discussed later.

TABLE 52 LACK OF EFFECT OF COMPLICATING ILLNESS ON PLASMA GOLD LEVEL

Group	Number of Samples	Mean Observed Plasma Gold mcg/ml	Mean Dose Rate mg/wk	Mean Adjusted Plasma Gold[1] mcg/ml
No Complicating Illness	141	2.03	12.6	1.99
Endocrine or Endocrine + other complicating Illness	24	2.48	16.6	1.74
Other Complicating Illness	78	1.40	10.6	1.70
Null hypothesis: no difference between groups[2]				
F		5.0 p<0.01 df 2/240	4.9 p<0.01 2/240	2.6 ns 2/239

[1] Plasma gold level is adjusted by covariance to 12.4 mg/wk dose rate.

[2] Null hypothesis is rejected if level of signifcance, p≤ 0.05 for F. When null hypothesis is upheld ns, not significant is indicated with F.

TABLE 53 LACK OF EFFECT OF SALICYLATE THERAPY ON PLASMA GOLD LEVELS

Group	Number of Samples	Mean Observed Plasma Gold mcg/ml	Mean Dose Rate mg/wk	Mean Adjusted Plasma Gold[1] mcg/ml
Gold	21	1.87	14.4	1.56
Gold + Salicylate	174	1.83	12.3	1.87
Null hypothesis:no difference between groups[2]				
F		0.008 ns df 1/193	1.09 ns 1/193	1.97 ns 1/192

[1] Plasma gold level is adjusted by covariance to 12.5 mg/wk dose rate.

[2] Null hypothesis is upheld when level of significance, $p>0.05$ for F and indicated ns.

TABLE 54 LACK OF EFFECT OF SIDE EFFECTS ON PLASMA GOLD LEVELS

Group	Number of Samples	Mean Observed Plasma Gold mcg/ml	Mean Dose Rate mg/wk	Mean Adjusted Plasma Gold[1] mcg/ml
No Side Effects	87	2.47	15.5	1.95
Samples taken prior to Side Effects	38	2.52	16.9	1.78
samples taken during Side Effects	13	2.65	14.2	2.38
Samples taken after Side Effects subsided	99	1.10	8.0	1.88
Null hypothesis:no difference between groups[2]				
F		14.2 $p<0.005$ df 3/233	20.6 0.005 3/233	1.3 ns 3/232

[1] Plasma gold level is adjusted by covariance to 12.5 mg/wk dose rate.

[2] Null hypothesis is rejected if $p<0.05$ for F. When null hypothesis is upheld ns is indicated with F.

D. RESULTS CORTISOL BINDING STUDIES

For each experimental group studied, four types of information on cortisol were available: (1) cortisol binding capacity of transcortin (P1), (2) cortisol binding affinity of transcortin (K1), (3) amount of unbound cortisol in the diluted plasma sample (U as mcg% or %), (4) total endogenous cortisol level in the plasma sample. (1) and (2) were calculated as described in literature survey and theoretical considerations (Section D (i)); two methods of calculation, the limit method and the mathematical method, were used so that two estimates of each binding parameter were obtained. Unbound cortisol was calculated from the 'zero added' cortisol point on the binding curve, and was corrected for adsorption efficiency. It must be kept in mind that unbound cortisol was determined at 8°C and in diluted plasma so it does not represent the amount of unbound cortisol circulating in the blood. A comparison of endogenous cortisol levels was also made for those patients selected for use in the binding studies. Albumin binding capacity (P2) and binding affinity (K2) were determined but, as seen in the Introduction and the Methodology section were inaccurate and imprecise. With highly variable data no differences would be detected, therefore albumin binding data has not been included.

(i) CORTISOL BINDING PROPERTIES OF PLASMA FROM NOVICE GOLD PATIENTS (GROUP A)

The data for the novice gold patients was analyzed as a randomized complete block experiment with patients forming blocks and stage of gold therapy as treatment. No significant differences were found in the cortisol binding capacity of transcortin, in the cortisol binding affinity of transcortin, in the total endogenous cortisol in plasma, in the amount of unbound cortisol (U mcg%) or in the fraction of cortisol unbound (U %) (Tables 55-57).

TABLE 55 TRANSCORTIN BINDING CAPACITY[1] OF NOVICE GOLD PATIENTS (GROUP A)

Patient	Stage of Gold Therapy		
	Pre-gold	First Gold Dose	Plasma Gold >2 mcg/ml
35	32.0 (23.2)	27.0 (21.5)	30.4 (19.6)
8	29.9 (23.2)	37.0	38.3 (26.7)
25	26.6 (19.3)	34.8 (26.8)	34.3 (27.1)
26	131.6 (30.1)	(26.2)	24.2 (24.8)

F blocks	1.26 ns (1.67 ns)
treatments	0.010 ns (0.57 ns)
Error mean Square	12.94 (956.54)

[1] P1 by limit method, () P1 by mathematical method, mcg%.

TABLE 56 TRANSCORTIN BINDING AFFINITY[1] OF NOVICE GOLD PATIENTS (GROUP A)

Patient	Stage of Gold Therapy		
	Pre-gold	First Gold Dose	Plasma Gold >2 mcg/ml
35	3.30 (5.25)	4.97 (4.62)	2.44 (20.26)
8	3.50 (37.6)	3.40 (15.5)	3.83 (8.32)
25	4.30 (4.71)	4.02 (193.49)	3.81 (7.35)
26	5.18 (60.16)	(1.92)	4.30 (3.82)
F blocks	2.26 ns (0.55 ns)		
treatments	1.27 ns (0.53 ns)		
Error Mean Square	7.89 (3719.17)		

[1] K1 by limit method, () K1 by mathematical method, l/m $x10^8$.

TABLE 57 PLASMA CORTISOL LEVELS[1], TOTAL AND UNBOUND, OF NOVICE GOLD PATIENTS (GROUP A)

Patient	Stage of Gold Therapy Pre-gold	 First Gold Dose	 Plasma Gold >2 mcg/ml
35	31.2 (8.5) [27.2]	14.4 (2.3) [16.1]	25.4 (6.8) [26.8]
8	25.6 (5.9) [23.2]	27.1 (5.9) [21.9]	22.6 (10.2) [45.0]
25	19.2 (4.4) [23.2]	25.3 (4.5) [17.6]	23.7 (4.8) [20.1]
26	39.4 (11.0) [33.0]	35.5 (9.9) [27.8]	24.0 (6.5) [27.2]

F blocks 1.54 ns (1.89 ns) [1.39 ns]
treatments 0.59 ns (0.59 ns) [1.71 ns]
Error Mean Square 42.34 (6.12) [48.02]

[1] Total endogenous cortisol, mcg%, () unbound cortisol mcg%, [ ] unbound cortisol expressed as % of total cortisol.

(ii) CORTISOL BINDING PROPERTIES IN MATCHED PATIENTS (GROUP B)

In this series of patients, variation due to difference in age, sex, diagnosis, drug therapy and complicating illness were minimized by matching patients in the gold and non-gold groups; thus differences in cortisol binding or plasma cortisol levels should be detected if present. The data was analyzed as paired 't' test. Again no significant differences were observed between the groups for any of the measured parameters (Tables 58-60).

TABLE 58 TRANSCORTIN BINDING CAPACITY[1] OF MATCHED PATIENTS (GROUP B)

Gold		Non-gold	
Patient	P1	Patient	P1
2	24.0 (20.5)	63	15.6 (11.5)
22	31.0 (17.8)	59	15.4 (11.1)
30	26.4 (17.7)	65	14.2 (12.1)
33	22.0 (14.3)	60	20.6 (15.9)
35	29.7 (20.9)	14	43.0 (17.1)
36	20.4 (13.8)	70	36.8 (28.4)
38	23.1 (14.8)	69	19.8 (14.7)
38	(15.7)	69	19.8 (14.7)
42	51.8 (25.7)	61	10.5 (9.4)
44	25.4 (17.8)	64	20.6 (13.1)
mean	28.2 (17.9)		21.8 (14.8)
standard deviation	9.5 (3.7)		10.9 (5.3)
Difference		6.4 (3.1)	
Standard deviation		16.9 (8.0)	
t		1.13 ns (1.21 ns)	

[1] P1 by limit method, () P1 by mathematical method, mcg%.

TABLE 59 TRANSCORTIN BINDING AFFINITY[1] OF MATCHED PATIENTS (GROUP B)

Gold Patient	K1	Non-gold Patient	K1
2	5.56 (3.22)	63	2.61 (14.16)
22	2.21 (4.63)	59	3.70 (15.90)
30	2.13 (2.88)	65	3.73 (4.99)
31	4.20 (5.74)	60	5.88 (6.81)
35	2.52 (27.97)	14	2.17 (5.58)
36	3.17 (4.91)	70	2.81 (4.63)
38	3.79 (9.13)	69	2.95 (38.90)
38	(21.3)	60	(38.90)
42	2.76 (12.97)	61	10.62 (8.13)
44	2.61 (4.52)	64	4.42 (2.95)
mean	3.22 (9.73)		4.32 (14.01)
standard deviation	1.12 (8.56)		2.61 (13.70)
difference		1.10 (4.29)	
standard deviation		2.99 (14.04)	
t		1.10 ns (0.966 ns)	

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[1] K1 by limit method, () K1 by mathematical method, 1/m x10^{8}.

TABLE 60 PLASMA CORTISOL LEVELS[1], TOTAL AND UNBOUND, OF MATCHED PATIENTS (GROUP B)

Patient	Gold Cortisol Level	Patient	Non-gold Cortisol Level
2	23.0 (6.0) [25.9]	63	8.6 (3.1) [36.1]
22	6.9 (3.1) [45.5]	59	2.2 (0.5) [23.8]
30	24.2 (8.5) [35.2]	65	7.7 (2.3) [30.2]
31	15.0 (3.8) [25.6]	60	20.2 (5.2) [25.9]
35	24.4 (9.2) [37.9]	14	26.5 (14.2) [53.7]
36	8.8 (2.2) [25.3]	70	23.2 (5.3) [23.0]
38	8.4 (1.2) [19.9]	69	13.7 (4.2) [31.0]
38	13.4 (4.6) [34.2]	69	13.7 (4.2) [31.0]
42	16.1 (7.1) [43.9]	61	1.6 (0.2) [13.5]
44	23.8 (6.6) [27.9]	64	14.6 (3.3) [22.3]
mean	16.4 (5.3) [32.1]		13.2 (4.3) [29.0]
standard deviation	7.0 (2.6) [8.6]		8.4 (3.9) [10.7]
Difference		3.2 (1.0) [3.1]	
standard deviation		10.3 (4.0) [14.3]	
t		0.98 ns (0.80 ns) [0.68 ns]	

[1] Total endogenous cortisol, mcg%; () unbound cortisol, mcg%; [ ] unbound cortisol expressed as % of total cortisol.

(iii) CORTISOL BINDING PROPERTIES IN PATIENTS GROUPED ACCORDING TO PLASMA GOLD LEVEL (GROUP C)

Variation in the amount of gold in plasma is considered in this series of patients. The data was initially analyzed by analysis of variance randomized design with unequal replication, using a least significant difference (lsd) test to detect differences (Steel, 1960). Caution must be used in interpreting differences detected by the lsd when F is non-significant because multiple comparisons are made with the lsd and false differences may be detected. Cortisol binding capacity was elevated at plasma gold levels of 5 mcg/ml or greater (Table 61) when plasma gold was grouped into 9 groups or when adjacent groups were combined giving 5 groups. The latter grouping puts more data in each group and makes the test more powerful in detecting differences. Because patients do not occur uniformly throughout all plasma gold groups, an effect due to patient difference not treatments could produce this effect. When only data for patients replicated at several plasma gold levels was used in analysis of variance complete block design with patients as blocks, the difference was no longer significant (Table 62). The effect on transcortin binding capacity is not due to gold treatment.

The difference between K1 (mathematical) for non-detectable gold and no gold is due to bias introduced by one large value in the former group. The difference does not occur by the limit method, so is probably not real (Table 63). No other differences in K1 were found. Endogenous cortisol levels and unbound cortisol, mcg%, were low in group 4-<5 mcg/ml and high in group >6 mcg/ml; however each group consisted of only two patients so the meaningfulness of this difference is questionable (Table 64). When the data was pooled into 5 groups, as described before, these differences disappeared indicating that they were due to chance. No other differences were found in total plasma cortisol or unbound cortisol.

TABLE 61 TRANSCORTIN BINDING CAPACITY[1] FOR PATIENTS GROUPED BY PLASMA GOLD LEVEL (GROUP C)[2]

Plasma Gold Level mcg/ml	P1					mean[3]	
no gold	26.6	10.5	20.6	32.0	14.2	20.8[4] [5]	20.8
	(19.3	9.4	13.1	23.2	12.1)	(15.4)	(15.4)
not detectable	26.4	20.4	23.1	34.8	49.2	30.8[5]	28.4
	(17.7	13.8	14.8	26.8	27.4)	(20.1)	(18.8)
<1	21.7	21.9	27.0	37.0	22.0	25.9[7]	
	(15.9	17.4	21.5		14.3)	(17.3)	
1-<2	25.4	33.9	22.1	27.1		27.1	27.1
	(17.8	25.3	17.4	19.3)		(20.0)	(21.0)
2-<3	21.1	33.0	21.2	24.2	35.5	27.0	
	(17.6	24.8	16.2	24.8	25.4)	(21.8)	
3-<4	24.0	30.4	34.3	23.2		28.0	27.4
	(20.5	19.6	27.1	17.0)		(21.0)	(20.2)
4-<5	25.0	27.2				26.1	
	(16.0	21.0)				(18.5)	
5-<6	27.0	37.6	34.2	38.4		34.3[4] [7]	33.7
	(16.6	21.2	29.4	47.1		(28.6)	(27.4)
>6	37.6	27.6				32.6	
	(31.3	18.5)				(24.9)	

Overall mean all gold patients	28.8 (21.4)	
Standard deviation	6.9 (6.8)	
F	1.20ns (1.39ns)	2.31ns (2.75[6])
Error mean square	55.55 (43.85)	50.57 (39.72)

[1] P1 by limit method, () P1 by mathematical method, mcg%.

[2] Order of data is the same as in Table 33 where the patients are described.

[3] First value for 9 groups of data, second value for 5 groups of data: no gold, not detectable to <1, 1-<3, 3-<5, >5 mcg/ml.

[4] Significantly different by least significant difference (lsd); t=2.70 (2.96), p<0.05 (0.01), standard error of difference (sed) =5.00 (4.44).

[5] Significantly different by lsd; t=2.121 (1.12), p<0.05 (ns), sed=4.71 (4.19).

[6] p<0.05.

[7] Significantly different by lsd; t=1.68 (2.41), p ns (<0.05), sed=5.00 (4.68).

TABLE 62 TRANSCORTIN BINDING CAPACITY[1] OF PATIENTS GROUPED BY PLASMA GOLD LEVEL
ANALYSIS OF VARIANCE RANDOMIZED COMPLETE BLOCK DESIGN

Plasma Gold Level mcg/ml	Patient								
	25	35	38	44	54	2	11	46	Treatment Means
no gold	26.6	32.0							29.3
<1	34.8	27.0	23.1	21.9					26.7
1-<3	33.0		22.1	25.4	21.1			27.6	25.8
3-<5	34.3	30.4			25.0	24.0	27.2		28.2
≥ 5						38.0	30.7	34.2	34.3

F blocks	0.80 ns
treatments	1.07 ns
Error Mean Square	331.72

[1] P1 by limit method, mcg%.

TABLE 63 TRANSCORTIN BINDING AFFINITY[1] FOR PATIENTS GROUPED BY PLASMA GOLD LEVEL (GROUP C)[2]

Plasma Gold Level mcg/ml	K1	Mean[3]	
no gold	4.30 10.62 4.42 3.30 3.73	5.27[4]	5.27
	(4.71 8.13 2.95 5.25 4.99)	(5.21)	(5.21)
not detectable	21.3 3.17 3.79 4.01 10.11	4.64[4]	4.41
	(2.88 4.91 9.13 193.49 30.36)	(48.15)	(28.16)
<1	4.41 3.91 4.97 3.40 4.20	4.18	
	(6.92 8.07 4.62 15.50 5.74)	(8.17)	
1-<2	2.61 5.44 11.20 3.80	5.79	4.65
	(4.52 7.04 17.90 5.51)	(8.74)	(9.44)
2-<3	4.34 5.17 3.37 4.30 1.61	3.76	
	(15.04 13.27 14.38 3.82 3.42)	(9.99)	
3-<4	5.56 2.24 3.81 6.90	4.68	4.67
	(3.22 20.26 7.35 33.89)	(16.18)	(15.52)
4-<5	2.09 7.21	4.65	
	(5.31 23.11)	(14.21)	
5-<6	4.23 2.03 5.88 12.42	6.14	5.86)
	(9.53 4.90 7.71 4.65)	(6.70)	(7.91)
>6	7.24 3.38	5.31	
	(8.48 12.20)	(10.34)	
Overall mean all gold patients		4.81	
		(16.36)	
standard deviation		2.60	
		(33.79)	
F		0.305	0.320ns
		(0.822	0.676ns)
Error mean square		8.160	7.443
		(1036.3)	(1032.3)

[1] K1 by limit method, () K1 by mathematical method, 1/m x10^{8}.

[2] Order of data is the same as in Table 33 where patients are described.

[3] First value for 9 groups of data, second value for 5 groups of data: no gold, not detectable to <1, 1-<3, 3-<5, >5 mcg/ml.

[4] Significantly different by lsd; t=0.350 (2.110), p ns (<0.05), sed=1.81 (20.36).

TABLE 64 PLASMA CORTISOL LEVELS[1], TOTAL AND UNBOUND, FOR PATIENTS GROUPED BY PLASMA GOLD LEVEL (GROUP C)[2]

PLasma Gold Level mcg/ml	Cortisol Level					Mean[3]	
no gold	19.2	1.6	14.6	31.2	7.7	14.9	14.9
	(4.4	0.2	3.3	8.5	2.3)	(3.74)	(3.74)
	[23.2	13.5	22.3	27.2	30.2]	[23.3]	[23.3]
not detectable	24.2	8.8	8.4	25.3	21.4	17.6	16.6
	(8.5	2.2	1.7	4.5	1.0)	(3.6)	(3.4)
	[35.2	25.3	19.9	17.6	4.5]	[20.5]	[21.0]
<1	10.4	10.8	14.4	27.1	15.0	15.5	
	(2.2	2.4	2.3	5.9	3.8)	(3.3)	
	[21.6	22.0	16.1	21.9	25.6]	[21.4]	
1-<2	23.8	24.7	14.2	19.8		20.6	18.3
	(6.6	4.0	1.5	4.8)		(4.2)	(3.9)
	[27.9	16.2	10.4	24.4]		[19.8]	[21.1]
2-<3	7.00	23.2	12.3	24.0	15.7	16.4	
	(1.4	4.0	3.3	6.5	3.1)	(3.7)	
	[19.4	17.4	27.2	27.2	19.9]	[22.2]	
3-<4	23.0	25.4	23.7	10.1		20.6	16.8
	(6.0	6.8	4.8	1.4)		(4.8)	(3.7)
	[25.9	26.8	20.1	13.9]		[21.7]	[21.2]
4-<5	3.9	14.4				9.2[4]	
	(1.1	1.9)				(1.5)	
	[27.4	13.4]				[20.4]	

5-<6	10.3 (1.8 [17.3	18.4 4.6 25.1	22.7 3.4 15.0	30.3 2.2) 7.1]	20.4 (3.0) [16.1]	23.2 (4.0) [18.2]
>6	37.9 (7.4 [19.6	19.4 4.9) 25.0]			28.6[4] (6.2) [22.3]	
Overall mean all gold patients					18.4 (3.7) [20.5]	
standard deviation					7.8 (2.1) [6.6]	
F					1.01ns (0.73ns) [0.35ns]	0.86ns (0.08ns) [0.40ns]
Error mean square					68.112 (5.09) [50.64]	69.427 (5.35) [46.32]

[1] Total endogenous cortisol mcg%, () unbound cortisol mcg%, [ ] unbound cortisol expressed as a % of total cortisol.

[2] Order of data is the same as in Table 33 where patients are described.

[3] First value for 9 groups of data, second value for 5 groups of data: no gold, not detectable to <1, 1-<3, 3-<5, >5 mcg/ml.

[4] Significantly different by lsd; t=2.36 (2.06) [0.27], p<0.05 (<0.05) [ns], sed=8.25 (2.26) [7.12].

E. RESULTS QUANTITATION OF SKIN, HAIR, NAIL AND BLOOD GOLD LEVELS

Analysis of gold in skin biopsies was performed by flameless atomic absorption (AAS) and by neutron activation analysis (NAA). Where only neutron activation analysis was performed on a particular sample, expected AAS result was predicted by regression using the equation given in Table 10. Quantitation of gold in other specimens was done by flameless atomic absorption.

Skin gold levels were consistently higher than plasma gold levels (Table 65) in all patients receiving gold, indicating the skin is a storage area for gold. Nails showed higher levels of gold than plasma but not as much as skin. Hair gold was less than plasma gold, nail gold or skin gold, suggesting hair has a lesser tendency to accumulate gold. Red blood cells contained 14.9 to 47.0% of the concentration of the gold found in plasma, which is in keeping with the results of Lawrence (1961), Oka (1973), and Smith (1973).

TABLE 65 GOLD LEVELS OF SKIN, HAIR, NAILS, AND BLOOD

Patient	Total Gold mg Duration Therapy months	Dose Rate mg/wk	Last Dose & Time Prior to Biopsy	Plasma or Serum Gold mcg/ml	RBC Gold mcg/g	Skin Gold mcg/g		Hair Gold mcg/g	Nail Gold mcg/g
						NAA	AAS		
W	>2500		24 wk	0.603	0.154	65.041	70.992	0.631	3.485
51	2492.5 75	2.27	25 mg 11 wk	0.826[1]	0.206[2]	40.98	(52.780)[3]		
33	5675 110	5.00	25 mg 5 wk	0.923[1]	0.181[2]	100.00	139.962		
12	1967.5 60	17.50	25 mg 10 days	2.914	0.483	25.850	38.000	0.235	0.977
3	1770 39	6.25	25 mg 4 wk	2.024	0.938	12.251	15.010	0.710	3.955
31	962.5 24	5.00	25 mg 5 wk	0.672[1]	0.121[2]	8.200	(7.503)[3]		
23	1360 38	5.00	25 mg 5 wk	2.394	0.782	11.890	10.727	0.483	1.507

21	942.5 24	5.00	25 mg 5 wk	0.705[1]	0.393[2]	7.895	10.284
J	17.5					>0.155	0.364
N	17.5					0.480	(0) [3]

[1] serum.

[2] From clotted blood.

[3] AAS value predicted from NAA result.

Skin gold levels were higher in the chrysiasis patients than in the non-chrysiasis patients; however the chrysiasis patients had received more total gold. Skin gold levels for this group fell within the 95% confidence limits of the regression line (Steel, 1960) relating skin gold levels and total gold dose for non-chrysiasis patients (Figure 13), indicating that skin gold level was similarly related to total gold dose in both groups. Confidence limits are calculated as follows: $(s/\sqrt{n}) \times t(0.05)$, where s is standard deviation about regression, n is number of pairs of values used to determine the regression line, and t(0.05) is tabulated value for degrees of freedom n-2. This suggests any patient who receives a sufficiently large dose of gold would develop chrysiasis, in agreement with the observations of Schmidt (1941). The correlation of total dose and skin gold level is not significant therefore the confidence limits are quite large. Also, extrapolation of the regression line gives large confidence limits. This tends to discount the interpretation that skin gold level is simply related to total gold dose.

For non-chrysiasis patients skin gold levels were well correlated with dose rate (Figure 14). Skin gold levels in chrysiasis patients were not related in the same way to dose rate, since their values were outside the 95% confidence limit of the regression line of skin gold level versus dose rate for non-chrysiasis patients. The chrysiasis patients had higher skin gold levels than expected, based on dose rate. This suggests that chrysiasis patients may preferentially accumulate gold in the skin. If this is in fact true, plasma gold levels would likely be lower than expected from the dose of gold in the chrysiasis patients. This was in fact the case (Figure 15) when total gold dose was related to plasma gold level for non-chrysiasis patients. Plasma gold level was not correlated with dose rate in the non-chrysiasis patients so regression analysis is not warranted with this data.

When the renewal time of the skin, 13 to 18 days (Allen, 1967), is considered it may be more reasonable to expect that gold dose rate would determine skin gold level rather than total gold dose. This is supported by the stronger correlation between skin gold level and gold dose rate than between skin gold and total gold dose. Thus it appears that chrysiasis patients may preferentially store gold in the skin when compared to patients who do not exhibit chrysiasis, after considering differences in dose for the two groups.

Frequent skin biopsies from patients would be both ethically and practically unaccepatable so it was hoped that hair or nails would have the same levels of gold as skin or at least that skin gold levels were predictable from hair or nail gold; however this was not the case (Table 65) (correlations: skin and hair gold r=0.0123 ns, df=2; skin and nail gold r=0.213 n2, df=2).

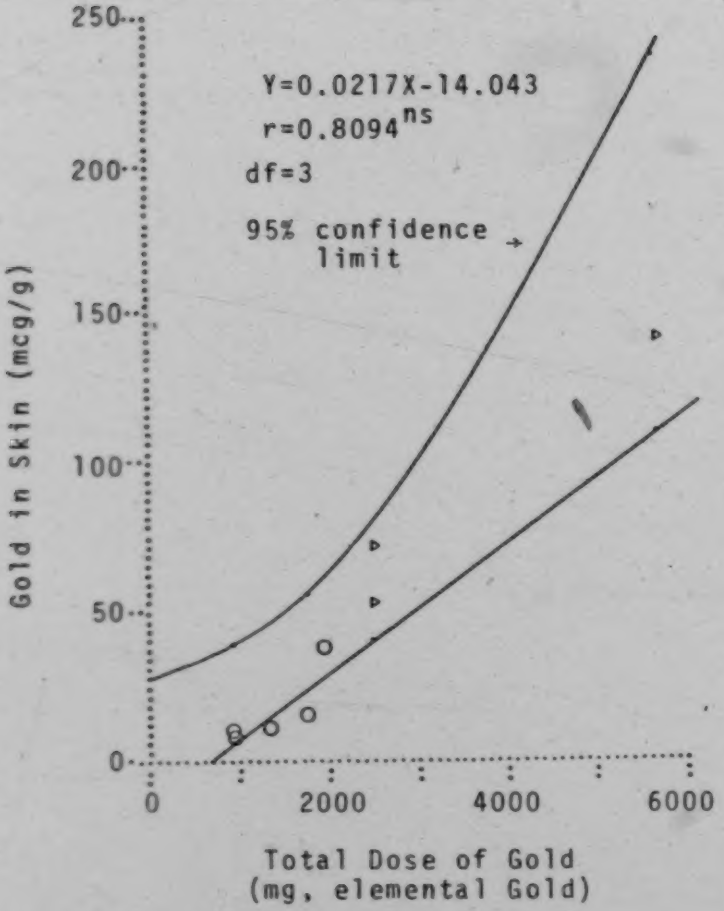

FIGURE 13 Relationship of Skin Gold Levels and Total Dose of Gold for Non-chrysiasis Patients.

▹ - Chrysiasis Patients
○ - Non-chrysiasis Patients

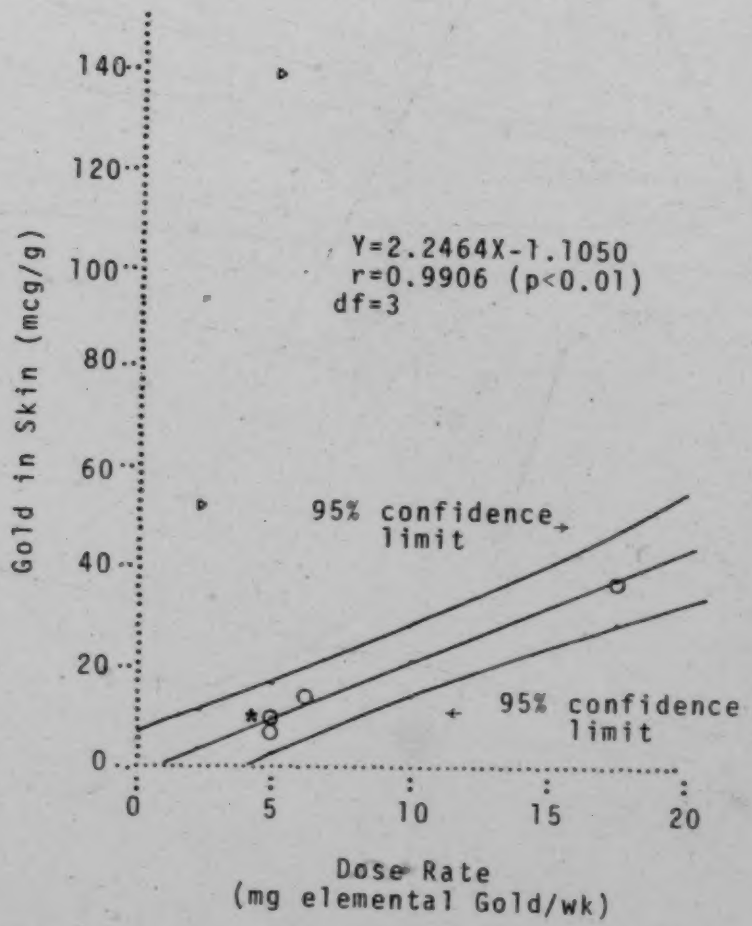

FIGURE 14 Correlation of Skin Gold Levels and Gold Dose Rate for Non-chrysiasis Patients.

▹ - Chrysiasis Patients
○ - Non-chrysiasis Patients

* - Two points

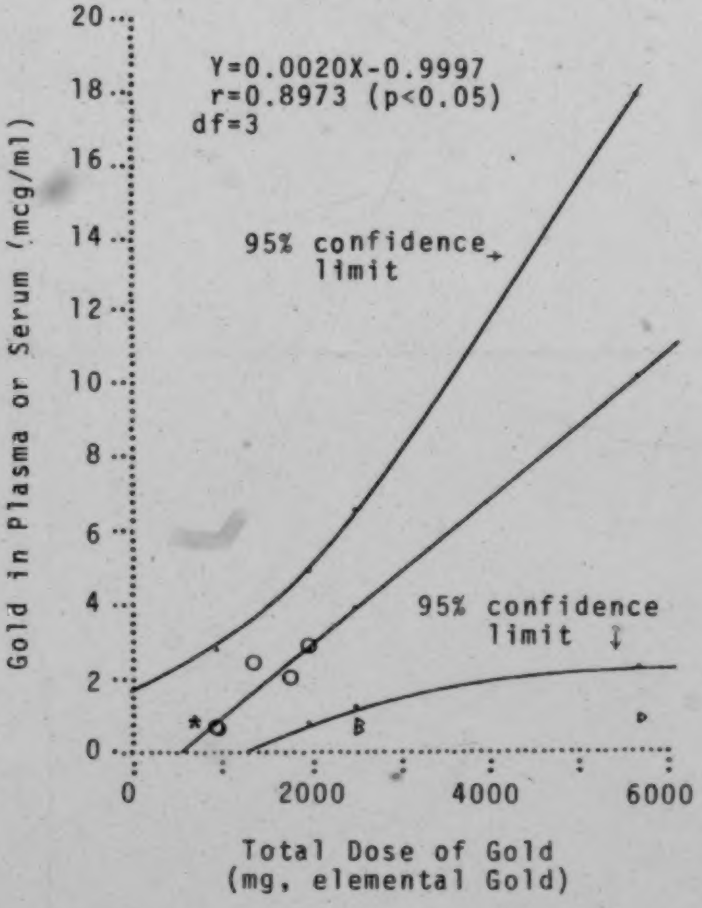

FIGURE 15 Correlation of Plasma or Serum Gold Levels With Total Dose of Gold for Non-chrysiasis Patients.

▹ - Chrysiasis Patients
o - Non-chrysiasis Patients

* - Two points

DISCUSSION

A. ANALYSIS OF GOLD

Analysis of gold by atomic absorption spectroscopy offers a rapid, specific and sensitive method for determination of gold in biological samples. Flame atomic absorption has a sensitivity which is sufficient for the analysis of gold in plasma of patients on an active regimen of gold therapy, but the sensitivity is inadequate for prolonged studies after the administration of gold is stopped. Flameless atomic absorption offers the advantage of sufficient sensitivity for these prolonged studies, and also the advantage of the ability to measure gold in small tissue samples. The gold content of most tissue samples in this study was sufficiently high to require dissolution and dilution of tissue samples; thus it was not necessary to use the maximum sensitivity of the flameless atomic absorption method. Theoretically, because of the three cycle process in this flameless AAS, tissue samples could be analyzed directly without prior digestion of organic material. Attempts to analyze tissue (approximately 1 mg) directly were not satisfactory because of the incomplete destruction of organic material during the 'char' stage. Despite the fact that deuterium background correction was used, some background absorption occurred during the atomize phase. Also smoking during the atomize phase attenuated the gold line sufficiently to reduce sensitivity 60-80%. Prolonged ashing at moderate temperatures did not completely destroy the organic matrix, and since most of the ashing occurred in the first few seconds prolonged ashing would not solve the problem. Higher ashing temperatures were also insufficient to destroy all organic material. At these temperatures some atomization of the gold occurred prior to analysis causing poor reproducibility. It was found that the gold signal occurred before the smoking in the atomize phase so it would be possible to distinguish the gold signal from the background. The use of smaller samples would alleviate this problem, but then the additional problem of accurate weighing of micrograms of tissue arises. With a suitable microgram balance this is possible but may be only slightly less tedious than digestion of tissue prior to flameless AAS analysis. For the purpose of this study digestion of the tissue, dilution and analysis of aliquots gave sufficient sensitivity and had the added advantage that several replicate determination could be made on each sample.

It was shown by flame AAS that the chemical form of gold (ionic or covalent) did not affect the AAS response. The only problem with flame AAS was that of the viscosity of plasma which reduced the aspiration rate and thus the sensitivity of the method. Dilution of the plasma with surfactant only partially overcame this problem. For comparison with aqueous standards a correction for detection efficiency was required. The other alternative was to make the standard curves using blank plasma to which known amounts of gold had been added.

Neutron activation analysis of gold also provides a sensitive and specific means of analyzing gold in tissues; however unavailability of thermal neutron sources makes the method slow and inconvenient for many laboratories including this one. Complete analysis including counting was done by commercial activation analysis services in this study. Counting equipment suitable for activation analysis is available here but the problems associated with shipment of radioactive materials of relatively short half life made counting in our own laboratory impractical.

The two methods of gold analysis in tissue samples, flameless AAS and NAA, gave results which were well correlated but the AAS method gave higher results. Because the results are well correlated the difference cannot be due to a background or matrix effect. Analysis of samples of known gold content by NAA gave good results. The amount of gold found by NAA was correlated with the known gold content of the plasma samples. The slope of the regression equation was less than one but not sufficiently to account for the difference in NAA and AAS results. Comparison of NAA and flameless AAS for gold in biological samples has not been reported in the literature.

B. ANALYSIS OF PLASMA CORTISOL AND PLASMA BINDING OF CORTISOL

Interference studies supported the specificity of the CPB assay for cortisol from the point of view of non-steroid interference. Many of the compounds studied have not been previously investigated for their interference with the assay (Noujaim, 1971). Prednisone was found to interfere to a similar extent as has been reported elsewhere (Nugent, 1966). Prednisolone, a metabolite of prednisone, also interferes in CPB assay of cortisol but to a greater extent than prednisone, being detected almost as strongly as cortisol (Noujaim, 1971).

Determination of the cortisol protein binding parameters of plasma by competitive adsorption has been reported by other workers (Heyns, 1967; Pegg, 1969; Rousseau, 1972). The method used in the present study removes most of the ambiguity involved in the previous methods as to temperature of the determination, and reduces the dissociation of cortisol-protein complex during the adsorption step by working at 8°C.

Of the two methods of resolving binding data, the limit method gave results closer to those previously reported in the literature, gave more accurate results with test data, and gave more precise results for most parameters. The mathematical method should give truer estimates of binding parameters because it does not require the assumptions and approximations needed to resolve the binding curves as is the case with the limit method. The mathematical method is very sensitive to small errors in the data and can give grossly erroneous results for some sets of data. The limit method, being less sensitive to small errors in the data, gives more reasonable estimates of binding parameters with most data. These two methods have the advantage that they can be run using a small computer. 'Literature' binding parameters have been determined by a variety of approaches, many of which are only approximate methods; therefore literature values do not themselves represent absolute standards for the cortisol binding parameters of plasma. The methods used here were reasonably reliable with regard to transcortin binding parameters; albumin binding parameters were not determined accurately. It should be possible to determine both binding parameters in a single experiment by use of higher cortisol concentrations (500-1000 ng/ml) in the albumin part of the curve.

The values of unbound cortisol are high compared to normal physiological levels of 10% total cortisol being unbound (Beisel, 1964a). This is because dilution of plasma results in decreased binding. Because determinations are made at a lowered temperature, binding is increased due to increased transcortin affinity. Both these factors make direct extrapolation of unbound cortisol levels determined in the study to unbound cortisol circulating in the blood of patients impossible.

In this study estrogen treated females had elevated transcortin binding capacities as well as elevated total and unbound cortisol levels. This is contrary to the accepted idea that unbound cortisol is not elevated in these persons. The findings in the present study agree with those of Lindholm (1973).

C. EFFECT OF GOLD THERAPY ON PLASMA CORTISOL LEVELS AND PLASMA CORTISOL BINDING

The first phase of the patient investigations show that gold therapy does not alter circulating levels of plasma cortisol. Because of the size of the patient population available, a relatively unstructured group of data was available; however analysis of the data classified in several ways allowed control of certain variables. The timing of sampling from the patients relative to gold dosing allowed for observation of persistent rather than transient effects of gold on plasma cortisol. Because of the lag between dosing with gold and sampling from the patients, measurement of plasma gold levels are more meaningful in assessing response of plasma cortisol relative to the amount of drug administered. There is also the advantage that differences in absorption and distribution of gold between different patients are eliminated. Initially a sex difference in the response of plasma cortisol to gold was suspected since a significant correlation of plasma cortisol and plasma gold was found in male but not female patients. Post menopausal women did not, however, show this relationship. In a single female case (49) who had undergone oophorectomy no trend of increased cortisol with increased plasma gold was seen. Since low correlations in large groups of data can be significant but sometimes misleading, the data for plasma cortisol and plasma gold was grouped by plasma gold level and examined by analysis of variance. The same result of higher cortisol levels at high plasma gold levels (greater than 4 mcg/ml) was obtained; however closer examination of the data revealed that one male patient (2) was unduly important to the data for high plasma gold levels. Only one sample was taken from this patient at a plasma gold level less than 4 mcg/ml. In this sample, plasma cortisol was lower than in samples for this patient at greater than 4 mcg/ml. This patient had consistently high cortisol levels compared to the overall mean for gold patients so this effect was probably not due to high gold levels. Selecting only data from patients who achieved several different plasma gold levels during the study allowed analysis of the data in such a way as to minimize the contribution of a single patient to the overall results. This revealed that plasma cortisol was not related to plasma gold level. Analysis of data for novice gold patients before and during gold therapy, and data for matched patients also supported the view that gold therapy does not alter plasma cortisol levels.

Concomitant drug therapy while receiving gold also complicated the picture. A literature search revealed that few of the drugs patients were taking are known to affect plasma cortisol levels. Prednisone is the most likely drug to effect plasma cortisol levels, especially when it is used, as it is in rheumatoid arthritis, for long term therapy. Few patients in the study were receiving prednisone or other forms of corticosteroid therapy and of those that were, plasma cortisol levels were not abnormally low, as would be expected from adrenal suppression, or abnormally high, as would be expected if prednisone or its metabolite, prednisolone, in the blood were interfering in the assay. These two opposing effects could cancel out any observable effect. In addition corticosteroid therapy is known to reduce hypothalamus-pituitary-adrenal reserve but may not necessarily depress basal levels of circulating cortisol. Statistical analysis of the small number of cases was not possible but these cases probably did not effect the overall data to any large extent.

Most patients in the study were receiving salicylate therapy. In the few gold patients who were not receiving salicylate no difference in plasma cortisol level could be detected from those receiving salicylate. The consensus from the literature suggests that salicylates slightly elevate plasma corticosteroids. This trend was seen in the salicylate only, or salicylate + gold patients versus controls subjects (no drugs), but this may have been due to differences in age, or state of health between the controls and the patients receiving salicylates. Most of the other drugs used by the patients do not effect plasma corticosteroid levels. Examination of their effect in this study was not possible because of the small number of patients taking each drug. None of these other drugs interfered in the assay of cortisol.

A toxic reaction to any drug would result in stimulation of HPA axis and increased plasma cortisol levels. Any effect of gold therapy on plasma cortisol must be distinguished from a toxic effect. There was no effect on plasma cortisol related to the therapeutic effect of gold; but an effect on plasma cortsiol related to toxicity was observed. The samples taken from patients after an adverse reaction to gold had lower plasma cortisol levels than samples from patients who experienced no toxic reaction to gold. There was no trend to higher plasma cortisol levels in samples taken during adverse reactions. Relative depletion of adrenal corticosteroids in response to a toxic reaction could account for the lowered plasma cortisol levels after toxicity.

There was a slight trend toward higher plasma cortisol levels with advancing age in the group I patients. Similar reports in the literature of higher plasma corticosteroids in older persons have been made (Samuels, 1956; Querido, 1967) but the effect is very slight (not statistically significant) and has not been demonstrated in all studies (Pincus, 1962; Gherandacho, 1967).

The method of analyzing plasma cortisol is specific in the sense that it is free of non-steroid interferences. The various corticosteroids are assayed to varying extents. The introduction of the carbon tetrachloride water partitioning step improves the specificity for a polar steroid such as cortisol (Jeffery, 1971). Circulating metabolites of cortisol are more polar than cortisol so would not be separated by this step. Tetrahydrocortisol, 6-beta-hydroxycortisol and several other hydroxylated cortisol metabolites do not bind significantly to transcortin relative to cortisol so would not be assayed by this method (Ghosh, 1971). Any changes in the circulating cortisol metabolites would likely not affect the assay of cortisol. Thus if the hypothesis of Kelley (1961) that salicylates increase plasma corticosteroids but also increase metabolism of corticosteroids were true for gold, this method of analysis of cortisol would not likely detect increased circulating levels of metabolites. In vivo study of metabolism of cortisol would require collection of urinary metabolites as well as circulating metabolites.

Cortisol levels in 'normal' individuals (group III) were lower than in either disease groups (group I or II). This may be due to one, or a combination, of three factors. Firstly, group III, was younger than groups I or II. There seems to be a weak relationship between advancing age and increased plasma cortisol levels. Secondly, chronic disease, acting as a stress, may explain the higher levels in groups II and III. Thirdly, the increase in cortisol in groups II and III could be due to drug therapy of the patients. Which factor is causing the effect cannot be determined, nor is it of major importance to this study. Most of the analysis of data was done without regard for group III since it does not serve as a suitable control group. The control group for this study of the effect of gold on plasma cortisol is really group II, a group of patients of similar age, diagnosis, and concomitant drug therapy exclusive of gold.

The degree of protein binding of cortisol is particularly important to the active level of cortisol in the blood (Beisel, 1964a), therefore the binding of cortisol to the plasma proteins was studied. Because of the high dissociation rate of cortisol-transcortin, bound cortisol is readily available. Paterson (1973) has shown that rapid disocciation from protein binding sites can occur in vivo making bound cortisol available to tissues. Thus the determination of K1 is particularly important, since changes in the strength of binding could markedly effect the availability of bound cortisol. No effect of gold on the binding of cortisol was observed in novice gold patients early in their course of gold treatment, or in gold and non-gold patients matched as to various characteristics. Again, as with the study of total plasma cortisol, differences were found in transcortin binding capacity when data was grouped by plasma gold level. Transcortin binding capacity appeared to be elevated at plasma gold levels greater than 5 mcg/ml; however this effect was again due to presence of patients with high P1 at high plasma gold levels who did not appear at low plasma gold levels. The effect on transcortin binding capacity was not due to gold. Other measures, K1 and fraction unbound, of plasma binding of cortisol were unaffected by gold therapy. Interference studies in CPB assay of cortisol using gold sodium thiomalate indicate that gold does not displace cortisol from its binding sites *in vitro*. It can be concluded that gold does not produce its beneficial effect in rheumatoid arthritis by alteration of circulating cortisol or its binding to transcortin.

D. PLASMA AND TISSUE GOLD LEVELS

Plasma gold levels were related to gold dose. Plasma gold levels were positively correlated to total dose of gold up to a total dose of 500 mg, then after 500 mg of gold plasma gold levels were negatively correlated to total dose in group I patients. This change in relationship between plasma gold level and dose probably reflects the change in dosage schedule made at 500 mg of gold. After 500 mg of gold dosing is gradually reduced in frequency to once per month from the previous schedule of dosing once weekly. This points out the need for consideration of temporal factors in expressing dose. From the available data in this study, expression of gold dose as dose rate (mg/wk) seemed to be the most useful. The correlation of gold dose with plasma gold was strongest where dose was expressed as dose rate. For dose rates, where comparisons with literature values could be made, plasma gold levels in this study were similar to previously reported values. Dose rate is not however the ideal expression of gold dose because this ignores accumulation of gold which occurs with repeated dosing at constant dose rate. Attempts to account for this accumulation effect, by use of dose rate and total dose together, for multiple regression, to predict plasma gold levels did not significantly improve the relationship derived between plasma gold level and dose. Sampling schedule was not so designed as to allow estimation of pharmacokinetic rate constants for absorption, distribution and elimination of gold.

Female patients were found to have higher plasma gold levels than male patients, a difference that could not be explained by a difference in dose rate. It is possible that females on average weighed less than males. This could explain the difference. Only incomplete information was available on patients' weights. In this smaller group of patients for whom weights were known plasma gold levels were not higher in females than in males. A second possible explanation of higher plasma gold levels in females compared to males is that females have a higher proportion of body fat than males (Bell, 1965) therefore water soluble drugs, such as gold sodium thiomalate, have a lower volume of distribution in females than in males. This would result in higher plasma gold levels in females.

No effect of concomitant drug therapy on plasma gold level could be demonstrated in this study.

The literature indicates there is controversy as to whether patients who show side effects to gold have higher levels of gold than those patients free of side effects. Because of the strong correlation between plasma gold and gold dose rate, it was possible, by analysis of covariance, to show that patients who developed side effects to gold had similar plasma gold levels for a standardized gold dose rate as did patients who did not develop side effects. This study supports the view that patients who develop side effects to gold do not handle gold differently from those patients who do not exhibit side effects. However, as plasma gold was the only parameter measured and no attempt was made to determine the pharmacodynamics of gold in each patient, this can only regarded as a tentative conclusion.

The incidence of side effect in this study was 63%, which is somewhat higher than the reported incidence of 5-50% (Meyler, 1968). This higher incidence of side effects probably results from close surveillance of the patients for side effects and the reporting of mild side effects. The entire gold therapy period for all patients was included in reports of side effects to gold. In some cases this represents therapy over a period of several years. Despite higher plasma gold levels females did not show an increased incidence cf side effects to gold.

In the smaller group of patients from whom skin biopsies were obtained, plasma gold was positively correlated with total dose despite the fact that total dose was greater than 500 mg. This is probably because time since the last dose of gold was much more uniform in these patients then in patients in group I. Dose rate was more strongly correlated with skin gold levels, than was total gold dose.

High levels of gold were found in the skin compared to plasma. These high levels of gold in the skin coupled with the fact that about 6-14 g of skin are sloughed off by the body each day (Allen, 1967) suggests that the skin may be an important storage or excretory site for gold. Microdistribution studies have shown that gold is present in the dermis (Zimmerli, 1929) and in the basal layer of the epidermis (Edstrom, 1959; Montgomery, 1967), a layer of the skin which is sloughed off. If gold is also lost with this skin a significant amount of excretion could occur. For example, patient 51 had 53 mcg/g gold in her skin. If uniform distribution of gold throughout the skin of the entire body is assumed, an assumption which finds some support from the work of Bogg (1958), it can be calculated that 6.8% of the total dose of gold this patient has received is currently in the patients skin (females have an average of 3200 g of skin, males have an average of 4800 g of skin, Allen, 1967). If an average of 10 g of skin is lost per day by this patient and this skin contained gold at the above concentration this would amount to a current daily excretion of 0.5 mg of gold or 3.5 mg/week, an excretion which is significant compared to the urinary and fecal excretion of gold reported in the literature, and compared to the current dose rate for this patient. Urinary and fecal excretion of gold varies between 0.2 and 1.3 mg/day (Davis, 1969; Gottlieb, 1971a; Jones, 1971; Kapelowitz, 1964; Mascarenhas, 1972; Sliwinski, 1966; Freyberg, 1942; Gottlieb, 1972b; Hartung, 1940b; Sorensen, 1970; Lorber, 1973; Lawrence, 1961). Table 66 shows similar calculations for the other patients. It is noteworthy that the predicted excretion of gold via the skin for chrysiasis patients 51 and 33 is greater than their current intake of gold.

TABLE 66 PREDICTED WEEKLY EXCRETION OF GOLD VIA THE SKIN AT TIME OF BIOPSY

Patient	Predicted Excretion[1] of Gold via Skin mg/wk	Skin[2] Excretion % of Dose Rate
51	3.71	169
W	4.97	
33	9.8	196
26	2.66	15.2
12	1.05	16.8
31	0.5	10.0
23	0.75	15.0
21	0.72	14.4

[1] Skin gold level from Table 65 in mcg/g x 10 g/d x 7 d/wk /1000 mcg/mg = predicted weekly excretion of gold via the skin, mg/wk.

[2] Predicted excretion via the skin, mg/wk, / dose rate from Table 65 expressed as %.

If a linear build up skin gold is assumed with increasing total dose in a relationship similar to that shown in Figure 13, it is possible to calculate the total amount of gold excreted by patient 51. Assuming that dose rate was relatively constant for this patient, the average skin gold level can be predicted from the skin gold level corresponding to a dose equivalent to half the total dose at the time of biopsy. Average skin gold level by this method is 18 mcg/g for patient 51. Excretion of gold via the skin for this patient over the entire 75 month duration of gold therapy can be calculated as (75 months x 30 d/month x 18 mcg gold/g skin x 10 g skin/d) = 405 mg. This corresponds to 16.2% of the total dose this patient has received. Table 67 shows similar calculations of excretion by this method for all patients.

TABLE 67 PREDICTED TOTAL EXCRETION OF GOLD VIA THE SKIN ASSUMING LINEAR BUILD UP OF GOLD IN SKIN FROM INITIATION OF GOLD THERAPY

Patient	TD/2 mg	Predicted[1] Average Skin Gold mcg/g	Excretion[2] via Skin mg	% of[3] Total Dose
51	1246.25	18	405	16.2
W	>1250	13.5	243	9.7
33	2837.5	47	1551	27.2
26	983.75	8	144	7.3
12	885	6	70	4.0
31	481.25	0	0	0
23	680	1	11	0.8
21	471.25	0	0	0

[1] Average skin gold level predicted from Figure 13 for TD/2, where TD/2 is total gold dose divided by 2.
[2] Total excretion via skin throughout total duration of therapy = predicted average skin gold x duration of therapy from Table 65 x 10 g/d.
[3] Predicted excretion / Total Dose expressed as %.

Figure 13 suggests that gold does not start to accumulate in the skin until after 600 mg of total gold. Theory dictates that (0,0) is a point on the regression line, excluding the slight amount of gold in the body due to envirnomental exposure, but it is not possible to measure either dose or gold concentration in this case so the exact points cannot be plotted. However, theory does not dictate that the 'x' intercept be zero; it may be greater than zero, as appears with our data, if gold does not immediately go into the skin. Perhaps saturation of other sites in the body is needed before gold appears in the skin, so there may be a threshold level above which detectable levels of gold are seen in the skin. In fact the confidence interval includes the point (0,0). Alternately, calculation of excretion via the skin considering this would use skin gold levels corresponding to a dose of (total dose at time of biopsy + 600)/2, which is 20 mcg/g for patient 51. Excretion of gold via the skin would only occur during the time from which the patient had received greater than 600 mg of gold. Thus excretion via the skin for patient 51 would be (63 months x 30d/month x 20mcg/g x 10 g/d) = 378 mg or 15.2% of the total dose this patient has received. Table 68 shows similar calculations of skin excretion of gold considering that at least 600 mg of gold is necessary before significant gold accumulates in the skin.

TABLE 68 PREDICTED TOTAL EXCRETION OF GOLD VIA THE SKIN ASSUMING LINEAR BUILD UP OF GOLD IN THE SKIN AFTER A TOTAL DOSE OF 600 MG OF GOLD

Patient	TD+600[1] / 2 mg	Predicted[2] Skin Gold mcg/g	Time Since[3] TD=600 mg	Excretion[4] via Skin mg	% of[5] TD
51	1546.25	20	63 months	378	15.2
W	>1550	20	4.5 years	324	13
33	3137.5	53	104 months	1654	29.1
26	1283.5	14	54 months	227	11.5
12	1185	12	33 months	119	6.7
31	781.25	5	18 months	27	2.8
23	980	8	29 months	70	5.1
21	771.5	5	15 months	23	2.4

[1] (Total dose from Table 65 + 600)/2.

[2] Average skin gold level predicted from Figure 13 using (TD + 600)/2.

[3] Time elapsed since patient received 600 mg total dose of gold.

[4] Average predicted skin gold x time since TD was 600 x 10 g/d.

[5] Predicted excretion / TD from Table 65 exppressed as %.

This 600 mg threshold figure may explain why previous investigators have found lower or insignificant gold in skin. In the studies by Gottlieb (1972a) and Bogg (1958) similarly high levels of gold in skin were demonstrated in patient who had received high total doses of gold. If the hypothesis that excretion of gold can occur via the skin, is correct excretion of gold in the skin would be fairly significant in all patients when compared to current dose rate and in the chrysiasis patients would be very large. Studies of gold excretion have indicated that excretion via urine and feces is insufficient to prevent a considerable accumulation of gold in the body. Excretion via other routes such as saliva is not significant. Excretion via or into the skin may represent another important excretory route for gold.

This is an interesting hypothesis but its validity depends on uniform distribution of gold throughout the layers of the skin, so that the gold levels in whole skin biopsies reflect accurately the levels of gold in the epidermis, the part of skin which losses cells by desquamation. Evidence from the literature, quoted earlier, showed by light microscopy that gold is present in both the dermis and the epidermis. Electron microscopy of skin tissues from patients in our own study, revealed no aggregates of gold in the skin section examined. From this, it can be inferred that gold is uniformly dispersed in the skin, probably as atoms or molecules, but not as colloidal aggregates. Other studies which would be possible to confirm the uniformity of gold distribution throughout the layers of the skin would be cutting thick sections (30 microns) through the layers of the skin and assaying the gold content of these sections by flameless AAS. This method of analysis is sufficiently sensitive to determine gold in this size of sample provided that the concentration is relatively uniform. Also with the small size of samples, smoking during the atomize phase of the analysis would not likely be a problem. The epidermis, which is 60-800 microns thick (Lewis, 1967), would give 2 to 3 sections, while the dermis, which is 540-5200 microns thick (Allen, 1967; Lewis, 1967), would yield several sections. Kinetic studies of the rate of appearance of gold in the skin, by serial biopsies at one month intervals, would also be useful. Smaller biopsies than those used in the current study could be used because of the sensitivity of the flameless AAS method. These smaller biopsies would make the procedure more acceptable to patients. Unfortunately fingernail and hair gold levels were not similar to skin gold levels nor were they correlated with skin gold levels, so these tissues could not be used as substitute biopsy specimens in this type of study. Chrysiasis is a rare condition, therefore accumulation of a large amount of data on gold levels in these patients is difficult. Perhaps because of the current practice of maintaining gold therapy indefinitely in patients who respond successfully to gold, more cases of chrysiasis will appear in the future.

The relationship between gold dose rate and skin gold level in non-chrysiasis patients was strong while the relationship between skin gold level and total dose was much weaker. The chrysiasis patients have significantly higher gold levels in skin than would be predicted from their gold dose rate. This suggests that these patients preferentially accumulate gold in the skin when compared to non-chrysiasis patients. The fact that plasma gold levels are lower than predicted from the total dose for chrysiasis patients also supports the suggestion that in chrysiasis patients more gold goes to the skin than to the rest of the body; however the extrapolation of gold versus total dose reduces the value of this correlation. Both patients W and 51 in the chrysiasis group had been off gold for considerably longer than the patients in the non-chrysiasis group. Thus the lower plasma gold levels would be expected for these patients because more time for the elimination of gold from the body was available. Patient 33 who had had a lapse of 5 weeks since the last gold injection had a plasma gold level intermediate between plasma gold levels for patients in non-chrysiasis group who had had a four to five week lapse since the last gold injection.

The patients from whom nail and hair clippings were obtained had all been receiving gold therapy for at least 38 months. The rate of growth of fingernail was reported at 0.105 mm/day and the average length of time for a nail to grow from the cuticle to the free end of the nail was 4.6 months (Bean, 1963). The rate of growth of normal adult scalp hair is 7.1-12.0 mm/month (Kopito, 1967). The long duration of gold therapy compared to the rate for nail and hair growth is sufficient time for gold to appear in these tissues. The level of gold in hair and nails of some gold treated patients (Table 65) were quite similar to gold levels detected in some individuals not treated with gold (Table 8). Kanabrocki (1968) reported similar levels of gold in fingernails of non-gold treated individuals as were found in this study. These levels of gold may result from environmental contamination with gold rather than from depositon of gold from within the body. Bate (1966) reported that externally applied gold adsorbs strongly to hair and is difficult to remove. Whether gold in hair and nails is externally deposited or deposited from within the body, these tissues do not seem to be a significant storage site for gold.

Patient W had been receiving penicillamine therapy for arthritis for approximately 6 months prior to skin biopsy. Penicillamine administration is known to increase urinary and fecal excretion of gold and also to increase plasma gold levels likely by mobilization of gold from sites of deposition in tissues. Patient W had a plasma gold level of 0.603 mcg/ml which seems high for a patient off gold for 6 months when compared to dose rate data from the large group of patients in the gold cortisol study. This may be due to mobilization of tissue gold. Skin gold for this patient was however higher than in patient 51 who had received a similar total dose of gold, so it does not appear that gold has been mobilized from the skin by penicillamine in patient W. Exclusion of patient W did not alter the intrepretation of the relationship of skin gold and plasma gold with dose so patient W did not bias the results.

In Schmidt's 1941 review of chrysiasis, attention was drawn to a possible predisposing effect of antecedent dermatitis to the development of chrysiasis. In the present study 3 chrysiasis patients had previous dermatitis, 1 did not, while 1 non-chrysiasis patient had previous dermatitis while 4 did not. A further case (27), from whom no biopsy was obtained also had previous dermatitis. This case has been included with the chrysiasis group. This number of patients is too small to assess whether previous dermatitis is associated with chrysiasis.

In the study reported here, all patient with chrysiasis were females, a fact which had been noted by Schmidt (1941). He pointed out that this may be due to cosmetic considerations since women are more likely to note skin coloration changes than are men.

All chrysiasis patients in this study had received a higher total dose of gold than 1260 mg and none of the non-chrysiasis patients had received more than 3880 mg of gold. These figure of total dose are those suggested by Schmidt (1941) as the doses below which chrysiasis never occurs, and above which chrysiasis always occurs, respectively.

Summary and Conclusions

1. Flame atomic absorption analysis of gold indicates that chemical form of gold does not affect atomic absorption response.

2. A method for flameless atomic absorption analysis of gold in tissues has been described which requires prior digestion of material with nitric acid, dilution and analysis of an aliquot of the sample. Sensitivity of 16 pg was achieved with a precision of 0.06 mcg/g (standard deviation) at the lower limit of sensitivity.

3. Several drugs have been shown not to interfere in CPB assay of cortisol.

4. Programs have been written for the resolution, by a small computer (8K memory), of two component binding data. Examination of test data reveals that although the mathematical method of resolving binding data is theoretically correct it loses its advantage over the approximate limit method because the former method is very sensitive to errors in data which inevitably occur with experimental data.

5. Studies with patients indicate that gold therapy does not effect the level of cortisol circulating in the blood nor does gold affect cortisol binding to transcortin.

6. Female patients were found to have higher plasma gold levels than male patients at equivalent gold dose rates.

7. Patients who experienced side effects to gold therapy did not have different plasma gold levels from patients free of side effects at equivalent gold dose rates; however plasma levels of cortisol were reduced in those patients who had experienced side effects relative to patients free of side effects.

8. Studies with gold patients receiving large total doses of gold suggest that patients who develop chrysiasis have a preferential tendency to accumulate gold in the skin compared to patients who do not develop chrysiasis.

9. Skin gold levels are higher compared to plasma gold levels in all patients receiving more than 950 mg of gold. It is suggested that excretion of gold via sloughing off with the skin may be a significant route of excretion of gold and may help to prevent or reduce the accumulation of gold in the body.

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APPENDIX I

PROGRAM FOR MATHEMATICAL RESOLUTION OF BINDING DATA

```
C:FOCAL-11,LFOCA-A

  1.01 E
  1.05 A !"N1",N1,"  N2",N2,!
  1.06 A !"%CBG",NP,"  VOL",ND;S NP=NP/100;S ND=(ND-.5)/NE
  1.07 F J=1,(N1+N2);A !U(J),B(J);S B(J)=B(J)/(NP*ND)
  1.08 S G=N1+N2
  1.20 F II=1,G-3;F JJ=II+1,G-2;F KK=JJ+1,G-1;F LL=KK+1,G;S R=0;D 2
  1.22 GOTO 3.12

  2.09 S NZ=0
  2.10 D 10
  2.25 S L=4;D 11;D 12
  2.30 S L=2;D 18;D 11;I (P)2.95
  2.50 I (K1)2.60,2.6;S SK=SK+K1;S QK=QK+K1↑2;S C2=C2+1
  2.60 I (K2)2.70,2.70,2.65
  2.65 I (NZ)2.70;S TK=TK+K2;S BK=BK+K2↑2;S C4=C4+1
  2.70 I (X(0))2.80,2.80,2.75
  2.75 I (X(1))2.80;S SP=SP+X(0);S QP=QP+X(0)↑2;S C1=C1+1
  2.80 I (X(1))2.95,2.95,2.85
  2.85 I (NZ)2.95;S TP=TP+X(1);S BP=BP+X(1)↑2;S C3=C3+1
  2.95 T %,!,K1,X(0),K2,X(1);T %1,II,JJ,KK,LL

  3.12 I (C1)3.65,3.65
  3.15 T %,!"P1,UG%",SP/(C1*10)
  3.20 T " +- ",(FSQT((QP-SP↑2/C1)/(C1-1)))/(10),"  N",C1
  3.24 I (C2)3.66,3.66
  3.25 T !"K1,L/M",SK*.362E+9/C2
  3.30 T " +- ",(FSQT((QK-SK↑2/C2)/(C2-1)))*.362E+9,"  N",C2
  3.34 I (C3)3.67,3.67
  3.35 T !"P2",TP/(C3*10)
  3.40 T " +- ",(FSQT((BP-TP↑2/C3)/(C3-1)))/10,"  N",C3
  3.49 I (C4)3.68,3.68
  3.50 T !"K2",TK*.362E+9/C4
  3.55 T " +- ",(FSQT((BK-TK↑2/C4)/(C4-1)))*.362E+9,"  N",C4
  3.60 C
  3.65 T !"NO DATA";GOTO 3.24
  3.66 T !"NO DATA";GOTO 3.34
  3.67 T !"NO DATA";GOTO 3.49
  3.68 T !"NO DATA";GOTO 3.60

 10.05 S V(1)=U(II)/B(II);S V(2)=-U(II);S V(3)=U(II)↑2/B(II);
 10.06 S V(4)=-(U(II)↑2);S V(5)=1
 10.10 S V(6)= U(JJ)/B(JJ); S V(7)=-U(JJ);S V(8)=U(JJ)↑2/B(JJ)
 10.11 S V(9)=-(U(JJ)↑2);S V(10)=1
 10.15 S V(11)= U(KK)/B(KK); S V(12)=-U(KK); S V(13)=U(KK)↑2/B(KK)
 10.16 S V(14)=-(U(KK)↑2);S V(15)=1
 10.20 S V(16)=U(LL)/B(LL); S V(17)=-U(LL);S V(18)=U(LL)↑2/B(LL)
 10.21 S V(19)=-(U(LL)↑2);S V(20)=1

 11.05 I (P)11.29;S V=0
 11.10 S N=L-1;S I=-1
 11.11 F K=0,N;S P(K)=K+1
 11.12 F J=0,N;F K=0,L;D 19
 11.14 S M=1E-25
 11.16 F J=0,N;F K=0,N;D 14
 11.17 S P(P)=0
 11.19 F K=0,L; S A(P+L*K)=A(P+L*K)/M
 11.20 F J=0,N;D 15
 11.22 S I=I+1
 11.23 I (I-N)11.14,11.26,11.14
 11.26 F J=0,N;F K=0,N;D 17
 11.29 R

 12.10 S P=X(1)↑2-4*X(3)
 12.40 I (P)12.7,12.6
 12.50 S K1=(X(1)+FSQT(P))/2;S K2=(X(1)-FSQT(P))/2
 12.55 I (K1-K2)12.8;I (K1)12.7;I (K2)12.9;R
 12.60 S K1=X(1)/2;S K2=K1;I (K1)12.7;R
 12.70 S R=-1;R
 12.80 S K1=K2;S K2=(X(1)+FSQT(P))/2;I (K1)12.7;I (K2)12.9;R
 12.90 S K2=2000/.362E+9;S NZ=-1;R

 14.05 I (P(J))0,14.3,14.1
 14.10 I (FABS(A(J+L*K))-FABS(M))14.3
 14.20 S M=A(J+L*K)
 14.22 S P=J;S Q=K
 14.30 R

 15.10 I (J-P)15.2,15.4,15.2
 15.20 S D=A(J+L*Q)
 15.30 F K=0,L;S A(J+L*K)=A(J+L*K)-A(P+L*K)*D
 15.40 R

 17.10 I (1E-25-FABS(A(J+L*K)))17.2;R
 17.20 S X(K)=A(J+L*L)

 18.10 I(P)18.3;S V(1)=K1;S V(2)=K2;S V(3)=X(0)
 18.20 S V(4)=K1*K2;S V(5)=V(4);S V(6)=X(2)
 18.30 R

 19.10 S V=V+1;S A(J+L*K)=V(V)
*
```

For print out of mean estimates only replace 2.95 with S NZ=NZ. Printing of all estimates approximately doubles the run time for 8 data to about 23 minutes.

MATHEMATICAL RESOLUTION OF TEST DATA
(6 FIGURE ACCURACY)

```
CC

Y1:4   Y2:4

TCDC:2.5    VOL:5
:0.3 :1.40583
:0.6 :2.77760
:1.9 :3.01030
:3.6 :3.61440
:5.1 :4.00000
:10.4 :5.26101
:15.6 :6.31074
:22.3 :7.68104
                                                                    Data
     K1 mL/ng          P1 ng/mL         K2 mL/ng          P2 ng/mL       Set Used
=  0.299996E+01=  0.133335E+03=  0.999065E-02=  0.111315E+04=  1=  2=  3=  4
=  0.299996E+01=  0.133335E+03=  0.999531E-02=  0.111232E+04=  1=  2=  3=  5
=  0.299997E+01=  0.133335E+03=  0.999475E-02=  0.111163E+04=  1=  2=  3=  6
=  0.299997E+01=  0.133335E+03=  0.999642E-02=  0.111145E+04=  1=  2=  3=  7
=  0.299998E+01=  0.133335E+03=  0.999761E-02=  0.111133E+04=  1=  2=  3=  8
=  0.299999E+01=  0.133334E+03=  0.999975E-02=  0.111113E+04=  1=  2=  4=  5
=  0.299999E+01=  0.133334E+03=  0.999856E-02=  0.111126E+04=  1=  2=  4=  6
=  0.299999E+01=  0.133334E+03=  0.999904E-02=  0.111121E+04=  1=  2=  4=  7
=  0.299999E+01=  0.133334E+02=  0.999951E-02=  0.111116E+04=  1=  2=  4=  8
=  0.299999E+01=  0.133334E+03=  0.999805E-02=  0.111131E+04=  1=  2=  5=  6
=  0.299998E+01=  0.133334E+03=  0.999904E-02=  0.111121E+04=  1=  2=  5=  7
=  0.299999E+01=  0.133334E+03=  0.999928E-02=  0.111118E+04=  1=  2=  5=  8
=  0.299999E+01=  0.133334E+03=  0.999999E-02=  0.111112E+04=  1=  2=  6=  7
=  0.299999E+01=  0.133334E+03=  0.999975E-02=  0.111114E+04=  1=  2=  6=  8
=  0.299999E+01=  0.133334E+03=  0.999951E-02=  0.111116E+04=  1=  2=  7=  8
=  0.300007E+01=  0.133332E+03=  0.100099E-01=  0.111022E+04=  1=  3=  4=  5
=  0.300004E+01=  0.133333E+03=  0.100017E-01=  0.111096E+04=  1=  3=  4=  6
=  0.300004E+01=  0.133333E+03=  0.100012E-01=  0.111101E+04=  1=  3=  4=  7
=  0.300004E+01=  0.133333E+03=  0.100007E-01=  0.111106E+04=  1=  3=  4=  8
=  0.300002E+01=  0.133333E+03=  0.999975E-02=  0.111114E+04=  1=  3=  5=  6
=  0.300002E+01=  0.133333E+03=  0.100002E-01=  0.111110E+04=  1=  3=  5=  7
=  0.300002E+01=  0.133333E+03=  0.999999E-02=  0.111112E+04=  1=  3=  5=  8
=  0.300003E+01=  0.133333E+03=  0.100007E-01=  0.111105E+04=  1=  3=  6=  7
=  0.300002E+01=  0.133333E+03=  0.100002E-01=  0.111110E+04=  1=  3=  6=  8
=  0.300002E+01=  0.133333E+03=  0.999975E-02=  0.111114E+04=  1=  3=  7=  8
=  0.299997E+01=  0.133335E+03=  0.999761E-02=  0.111134E+04=  1=  4=  5=  6
=  0.299998E+01=  0.133334E+03=  0.999904E-02=  0.111120E+04=  1=  4=  5=  7
=  0.299998E+01=  0.133334E+03=  0.999904E-02=  0.111120E+04=  1=  4=  5=  8
=  0.300002E+01=  0.133333E+03=  0.100005E-01=  0.111108E+04=  1=  4=  6=  7
=  0.300001E+01=  0.133334E+03=  0.100002E-01=  0.111109E+04=  1=  4=  6=  8
=  0.300000E+01=  0.133334E+03=  0.999975E-02=  0.111113E+04=  1=  4=  7=  8
=  0.300008E+01=  0.133332E+03=  0.100010E-01=  0.111104E+04=  1=  5=  6=  7
=  0.300003E+01=  0.133333E+03=  0.100002E-01=  0.111110E+04=  1=  5=  6=  8
=  0.300001E+01=  0.133334E+03=  0.999951E-02=  0.111117E+04=  1=  5=  7=  8
=  0.299992E+01=  0.133336E+03=  0.999904E-02=  0.111119E+04=  1=  6=  7=  8
=  0.300014E+01=  0.133331E+03=  0.100107E-01=  0.111003E+04=  2=  3=  4=  5
=  0.300008E+01=  0.133332E+03=  0.100021E-01=  0.111092E+04=  2=  3=  4=  6
=  0.300007E+01=  0.133332E+03=  0.100017E-01=  0.111096E+04=  2=  3=  4=  7
=  0.300008E+01=  0.133332E+03=  0.100012E-01=  0.111102E+04=  2=  3=  4=  8
=  0.300005E+01=  0.133333E+03=  0.999999E-02=  0.111112E+04=  2=  3=  5=  6
=  0.300005E+01=  0.133333E+03=  0.100005E-01=  0.111108E+04=  2=  3=  5=  7
=  0.300005E+01=  0.133333E+03=  0.100002E-01=  0.111110E+04=  2=  3=  5=  8
=  0.300006E+01=  0.133333E+03=  0.100010E-01=  0.111103E+04=  2=  3=  6=  7
=  0.300005E+01=  0.133333E+03=  0.100002E-01=  0.111110E+04=  2=  3=  6=  8
=  0.300004E+01=  0.133333E+03=  0.999975E-02=  0.111115E+04=  2=  3=  7=  8
=  0.299996E+01=  0.133335E+03=  0.999737E-02=  0.111137E+04=  2=  4=  5=  6
=  0.299998E+01=  0.133334E+03=  0.999904E-02=  0.111120E+04=  2=  4=  5=  7
=  0.299998E+01=  0.133334E+03=  0.999904E-02=  0.111120E+04=  2=  4=  5=  8
=  0.300004E+01=  0.133333E+03=  0.100007E-01=  0.111105E+04=  2=  4=  6=  7
=  0.300002E+01=  0.133333E+03=  0.999999E-02=  0.111112E+04=  2=  4=  6=  8
=  0.300000E+01=  0.133334E+03=  0.999975E-02=  0.111113E+04=  2=  4=  7=  8
=  0.300009E+01=  0.133332E+03=  0.100010E-01=  0.111104E+04=  2=  5=  6=  7
=  0.300006E+01=  0.133333E+03=  0.100002E-01=  0.111110E+04=  2=  5=  6=  8
=  0.300001E+01=  0.133334E+03=  0.999975E-02=  0.111114E+04=  2=  5=  7=  8
=  0.299989E+01=  0.133336E+03=  0.999880E-02=  0.111122E+04=  2=  6=  7=  8
=  0.299966E+01=  0.133337E+03=  0.999522E-02=  0.111157E+04=  3=  4=  5=  6
=  0.299975E+01=  0.133336E+03=  0.999785E-02=  0.111131E+04=  3=  4=  5=  7
=  0.299973E+01=  0.133336E+03=  0.999832E-02=  0.111[illegible]6E+04=  3=  4=  5=  8
=  0.299997E+01=  0.133334E+03=  0.100005E-01=  [illegible]107E+04=  3=  4=  6=  7
=  0.299993E+01=  0.133334E+03=  0.999975E-02=  0.111114E+04=  3=  4=  6=  8
=  0.299998E+01=  0.133335E+03=  0.999951E-02=  0.111116E+04=  3=  4=  7=  8
=  0.300014E+01=  0.133332E+03=  0.100012E-01=  0.111102E+04=  3=  5=  6=  7
=  0.300008E+01=  0.133333E+03=  0.100005E-01=  0.111108E+04=  3=  5=  6=  8
=  0.299995E+01=  0.13333[illegible]E+03=  0.[illegible]951E-02=  0.111116E+04=  3=  5=  7=  8
=  0.299968E+01=  0.133337E+03=  0.999880E-02=  0.111121E+04=  3=  6=  7=  8
=  0.300062E+01=  0.133329E+03=  0.100021E-01=  0.111094E+04=  4=  5=  6=  7
=  0.300049E+01=  0.133331E+03=  0.100010E-01=  0.111105E+04=  4=  5=  6=  8
=  0.300016E+01=  0.133333E+03=  0.999975E-02=  0.111115E+04=  4=  5=  7=  8
=  0.299931E+01=  0.133339E+03=  0.999856E-02=  0.111123E+04=  4=  6=  7=  8
=  0.299863E+01=  0.133340E+03=  0.999832E-02=  0.111124E+04=  5=  6=  7=  8
P1,UGS=  0.133334E+02 +- =  0.120386E-01   J=  0.700000E+02
K1,L/M=  0.103600E+10 +- =  0.000000E+00   J=  0.700000E+02
P2=  0.111117E+03 +- =  0.000000E+00   J=  0.700000E+02
K2=  0.361981E+07 +- =  0.000000E+00   J=  0.700000E+02*
```

```
                    MATHEMATICAL RESOLUTION OF TEST DATA
*GO                    (5% RANDOM ERROR IN B)

V1:4  V2:4

%CPG:0.5   VOL:5
  W     B
:0.3  :1.87
:0.6  :2.19
:1.9  :2.97
:3.6  :3.49
:5.1  :3.93
:10.4 :5.00
:15.6 :6.62
:23.3 :7.30

= 0.256919E+01= 0.192052E+03= 0.552486E-05=-0.987455E+07= 1= 2= 3= 4
= 0.255364E+01= 0.190011E+03= 0.552486E-05=-0.556552E+07= 1= 2= 3= 5
= 0.251206E+01= 0.174459E+03= 0.552486E-05=-0.500178E+07= 1= 2= 3= 6
= 0.249499E+01= 0.172250E+03= 0.552486E-05=-0.359541E+07= 1= 2= 3= 7
= 0.248655E+01= 0.171299E+03= 0.552486E-05=-0.295408E+07= 1= 2= 3= 8
= 0.237560E+01= 0.211249E+03= 0.552486E-05=-0.179198E+08= 1= 2= 4= 5
= 0.314959E+01= 0.133593E+03= 0.841582E-01= 0.198373E+03= 1= 2= 4= 6
= 0.301563E+01= 0.139394E+03= 0.115731E-01= 0.103139E+04= 1= 2= 4= 7
= 0.303969E+01= 0.135363E+03= 0.248795E-01= 0.512348E+03= 1= 2= 4= 8
= 0.277966E+01= 0.151965E+03= 0.552486E-05=-0.454364E+04= 1= 2= 5= 6
= 0.279219E+01= 0.151705E+03= 0.552486E-05= 0.759202E+05= 1= 2= 5= 7
= 0.290397E+01= 0.149502E+03= 0.552486E-05= 0.757178E+06= 1= 2= 5= 8
= 0.279020E+01= 0.151214E+03= 0.552486E-05= 0.165390E+06= 1= 2= 6= 7
= 0.297252E+01= 0.145658E+03= 0.504732E-03= 0.155660E+05= 1= 2= 6= 8
= 0.354275E+01= 0.120743E+03= 0.831065E-01= 0.311090E+03= 1= 2= 7= 8
= 0.219457E+01= 0.211990E+03= 0.552486E-05=-0.138672E+08= 1= 3= 4= 5
= 0.469925E+00= 0.227471E+04= 0.552486E-05=-0.172093E+09= 1= 3= 4= 6
= 0.316255E+02= 0.637784E+02= 0.116655E+00= 0.357278E+03= 1= 3= 4= 7
= 0.321024E+02= 0.636424E+02= 0.117279E+00= 0.356363E+03= 1= 3= 4= 8
= 0.429064E+01= 0.117450E+03= 0.209131E-01= 0.600940E+03= 1= 3= 5= 6
= 0.403379E+01= 0.121901E+03= 0.552486E-05= 0.176956E+07= 1= 3= 5= 7
= 0.422373E+01= 0.119400E+03= 0.152965E-01= 0.785695E+03= 1= 3= 5= 8
= 0.362924E+01= 0.130421E+03= 0.552486E-05= 0.521285E+06= 1= 3= 6= 7
= 0.413139E+01= 0.119908E+03= 0.125966E-01= 0.906558E+03= 1= 3= 6= 8
= 0.389999E+02= 0.623400E+02= 0.119569E+00= 0.356052E+03= 1= 3= 7= 8
= 0.177692E+01= 0.215300E+03= 0.552486E-05=-0.461585E+07= 1= 4= 5= 6
= 0.172458E+01= 0.215979E+03= 0.552486E-05=-0.336607E+07= 1= 4= 5= 7
= 0.169879E+01= 0.216341E+03= 0.552486E-05=-0.274468E+07= 1= 4= 5= 8
= 0.213029E+01= 0.179597E+03= 0.552486E-05=-0.719676E+06= 1= 4= 6= 7
= 0.230527E+01= 0.167376E+03= 0.552486E-05= 0.695514E+06= 1= 4= 6= 8
= 0.512992E+02= 0.610190E+02= 0.120731E+00= 0.357149E+03= 1= 4= 7= 8
= 0.297012E+01= 0.149652E+03= 0.552486E-05= 0.244601E+06= 1= 5= 6= 7
= 0.399149E+01= 0.123663E+03= 0.108268E-01= 0.100341E+04= 1= 5= 6= 8
= 0.225495E+00= 0.303351E+04= 0.552486E-05=-0.593667E+08= 1= 5= 7= 8
= 0.629637E+00= 0.524295E+03= 0.552486E-05=-0.100396E+08= 1= 6= 7= 8
= 0.203599E+01= 0.214951E+03= 0.552486E-05=-0.136715E+08= 2= 3= 4= 5
= 0.471675E+00= 0.123912E+04= 0.552486E-05=-0.535115E+08= 2= 3= 4= 6
= 0.130026E+00= 0.168772E+05= 0.552486E-05=-0.443057E+09= 2= 3= 4= 7
= 0.129012E+00= 0.171593E+05= 0.552486E-05=-0.447913E+09= 2= 3= 4= 8
= 0.719023E+01= 0.105739E+03= 0.310960E-01= 0.470272E+03= 2= 3= 5= 6
= 0.604912E+01= 0.114712E+03= 0.529289E-04= 0.219046E+06= 2= 3= 5= 7
= 0.674032E+01= 0.110554E+03= 0.201475E-01= 0.670779E+03= 2= 3= 5= 8
= 0.469040E+01= 0.125593E+03= 0.552486E-05= 0.952450E+06= 2= 3= 6= 7
= 0.612627E+01= 0.114009E+03= 0.149021E-01= 0.819582E+03= 2= 3= 6= 8
= 0.124796E+00= 0.191779E+05= 0.552486E-05=-0.486805E+09= 2= 3= 7= 8
= 0.147173E+01= 0.226036E+03= 0.552486E-05=-0.500731E+07= 2= 4= 5= 6
= 0.140053E+01= 0.228294E+03= 0.552486E-05=-0.373803E+07= 2= 4= 5= 7
= 0.136919E+01= 0.229401E+03= 0.552486E-05=-0.314640E+07= 2= 4= 5= 8
= 0.155775E+01= 0.194485E+03= 0.552486E-05=-0.820842E+06= 2= 4= 6= 7
= 0.205264E+01= 0.170806E+03= 0.552486E-05= 0.608566E+06= 2= 4= 6= 8
= 0.123377E+00= 0.214156E+05= 0.552486E-05=-0.541707E+09= 2= 4= 7= 8
= 0.291620E+01= 0.145424E+03= 0.552486E-05= 0.249575E+06= 2= 5= 6= 7
= 0.491269E+01= 0.121546E+03= 0.114465E-01= 0.968773E+03= 2= 5= 6= 8
= 0.214299E+00= 0.213001E+04= 0.552486E-05=-0.579017E+08= 2= 5= 7= 8
= 0.494964E+00= 0.533966E+03= 0.552486E-05=-0.953428E+07= 2= 6= 7= 8
= 0.495692E+00= 0.372617E+03= 0.552486E-05=-0.938326E+07= 3= 4= 5= 6
= 0.371095E+00= 0.4493[illegible]E+03= 0.552486E-05=-0.900627E+07= 3= 4= 5= 7
= 0.350290E+00= 0.469795E+03= 0.552486E-05=-0.897204E+07= 3= 4= 5= 8
= 0.490677E+00= 0.298310E+03= 0.552486E-05=-0.302324E+07= 3= 4= 6= 7
= 0.491742E+00= 0.296124E+03= 0.552486E-05=-0.198002E+07= 3= 4= 6= 8
= 0.121911E+00= 0.665630E+05= 0.552486E-05=-0.169772E+10= 3= 4= 7= 8
= 0.141602E+01= 0.165962E+03= 0.552486E-05=-0.988577E+05= 3= 5= 6= 7
= 0.295243E+01= 0.126991E+03= 0.985956E-02= 0.106661E+04= 3= 5= 6= 8
= 0.192949E+00= 0.115592E+04= 0.552486E-05=-0.237020E+08= 3= 5= 7= 8
= 0.239141E+00= 0.588679E+03= 0.552486E-05=-0.796790E+07= 3= 6= 7= 8
=-0.121799E-01=-0.655349E+02=-0.561537E+00=-0.573717E+00= 4= 5= 6= 7
= 0.351527E-01= 0.102490E+05= 0.552486E-05=-0.660342E+05= 4= 5= 6= 8
= 0.169297E+00= 0.965582E+03= 0.552486E-05=-0.168722E+08= 4= 5= 7= 8
= 0.195936E+00= 0.617035E+03= 0.552486E-05=-0.752002E+07= 4= 6= 7= 8
= 0.135022E+00= 0.697021E+03= 0.552486E-05=-0.676002E+07= 5= 6= 7= 8
P1,UGT= 0.123119E+02 +- = 0.294066E+01  N= 0.290000E+02
K1,L/M= 0.154229E+10 +- = 0.323431E+10  N= 0.690000E+02
P2= 0.120151E+04 +- = 0.500345E+04  N= 0.190000E+02
KP= 0.157355E+05 +- = 0.166154E+05  N= 0.190000E+02*
```

PROGRAM FOR LIMIT METHOD RESOLUTION OF BINDING DATA

```
C:FOCAL-11.LFOCA-A

  1.01 E
  1.02 T !"INPUT DATA AS UNBOUND &BOUND NG/ML EXPTL SOLN"!!
  1.05 A ! "N1",N1," N2",N2, !
  1.06 A ! "%CBG",NP," VOL",ND;S NP=NP/100;S ND=(ND-.5)/ND
  1.07 F J=1,(N1+N2);A ! BU(J),B(J);S B(J)=B(J)/(NP*ND);D 18
  1.08 S N=N1;S S=1;D 12;S K1=-B;S V(3)=A
  1.10 S SY=0;S SX=0;S SP=0;S QX=0;S NN=0
  1.12 S N=N1+N2;S S=N1+1;D 12; S K2=-B;S V(6)=A/K2

  2.10 D 10
  2.25 S L=2;D 11

  3.15 T %,! "P1,UG%",X(0)/10
  3.25 T !"K1,L/M",K1*.362E+9
  3.35 T ! "P2",X(1)/10
  3.50 T ! "K2",K2*.362E+9
  3.60 Q

 10.05 S V(1)=K1;S V(2)=K2
 10.06 S V(4)=1;S V(5)=1

 11.05 I (P)11.29;S V=0
 11.10 S N=L-1;S I=-1
 11.11 F K=0,N;S P(K)=K+1
 11.12 F J=0,N;F K=0,L;D 19
 11.14 S M=1E-25
 11.16 F J=0,N;F K=0,N;D 14
 11.17 S P(P)=0
 11.19 F K=0,L; S A(P+L*K)=A(P+L*K)/M
 11.20 F J=0,N;D 15
 11.22 S I=I+1
 11.23 I (I-N)11.14,11.26,11.14
 11.26 F J=0,N;F K=0,N;D 17
 11.29 P

 12.10 F I=S,N;D 16
 12.15 S B=(SP-SX*SY/NN)/(QX-SX↑2/NN)
 12.20 S A=SY/NN-B*SX/NN
 12.25 I (-B)12.50,12.45,12.45
 12.45 P
 12.50 S B=-2000/.362E+9;T !"#";S A=200000

 14.05 I (P(J))0,14.3,14.1
 14.10 I (FABS(A(J+L*K))-FABS(M))14.3
 14.20 S M=A(J+L*K)
 14.22 S P=J;S Q=K
 14.30 R

 15.10 I (J-P)15.2,15.4,15.2
 15.20 S D=A(J+L*Q)
 15.30 F K=0,L;S A(J+L*K)=A(J+L*K)-A(P+L*K)*D
 15.40 P

 16.10 S SX=SX+B(I);S SY=SY+BU(I);S NN=NN+1
 16.15 S QX=QX+B(I)↑2;S SP=SP+B(I)*BU(I)

 17.10 I (1E-25-FABS(A(J+L*K)))17.2;R
 17.20 S X(K)=A(J+L*L)

 18.10 S BU(J)=B(J)/BU(J)

 19.10 S V=V+1;S A(J+L*K)=V(V)
*
```

PROGRAM FOR TRANSFORMATION OF PROTEIN BINDING DATA
- COMPETITIVE ADSORPTION

```
*C-FOCAL,69CF
*
*01.01 E
*01.02 A !"# REPLICATE COUNTS",N
*01.04 A !"SP ACT CI/MM",SA;S SA=SA*0.222E+13/.362E+9
*01.06 A !"CBG %",V;S V=V/200
*01.08 A !"ENDO F UG%",HC;S HC=HC*V/.1
*01.10 T !"DPM/HALF ML CBG";F I=1,N;A D;S SD=SD+D
*01.12 S D=SD/N;T D
*01.14 A !"ADSORBENT G",W,"  ML",VO
*01.18 A !!"# DATA",M
*01.20 T !"ADSORP CONST   A NG/ML   DPM/HALF ML"
*01.22 F I=1,M;S SB=0;D 2
*01.24 T !!"  U        B   (NG/ML EXPTL SOLN)"
*01.26 F I=1,M;X;D 3
*01.28 T "                                        "
*01.30 F I=1,M;X;D 4
*01.32 T "                                        "
*01.36 A ! "SCALE FACTOR Y",Y,"  X",X
*01.40 F I=1,M;S BU(I)=FITR(BU(I)*Y+.5);S B(I)=FITR(B(I)*X+.5)
*01.42 S I=1;T !"*",#;D 6.05;D 6.06
*01.43 F I=2,M;D 6
*01.45 I (BU(M)-1)1.50;F J=1,BU(M);T !"*"
*01.50 T #;F JJ=1,60;T "-"
*01.64 Q
*
*02.01 A !Z,A(I);F J=1,N;A B;S SB=SB+B
*02.02 S B(I)=SB/N;S A(I)=A(I)/2
*02.05 S AD=Z*W/VO;D 5;R
*
*03.01 S T=HC+A(I)+D/SA
*03.02 S B(I)=T*B(I)/D;S U=T-B(I);S BU(I)=B(I)/U
*03.04 T !%8.03,U,B(I);X
*
*04.01 T !BU(I),B(I);X
*
*05.01 S B(I)=D-((D-B(I)*2)/AD)
*
*06.01 I (K-BU(I)-1)6.02;F J=1,K-BU(I);T !"*"
*06.02 T #
*06.05 F JJ=1,B(I);T " "
*06.06 T "X";S K=BU(I)
**
```

PROGRAM FOR TRANSFORMATION OF PROTEIN BINDING DATA
- EQUILIBRIUM DIALYSIS

```
*C-FOCAL,69CE
*
*01.01 E
*01.04 A ! "N",N
*01.05 A ! "SP ACT CI/MM",SA;S SA=SA*.222E+13/.362E+9
*01.07 A !"CBG %",V;S V=V/100
*01.09 A !"ENDO F UG%",HC;S HC=HC*V/.1
*01.11 T !"DPM/HALF ML CBG";F I=1,N;A D;S SD=SD+D
*01.13 S D=SD/N;A !"VOL INNER SOLN =OUTER SOLN",VI
*01.15 A !"NUMBER OF DATA",M
*01.17 T !!"A NG/ML   DPM PROTEIN    DPM OUTSIDE"
*01.19 F I=1,M;D 2;S SP=0;S SO=0
*01.21 T !!"   U          B  (NG/ML INNER SOLN)"
*01.23 F I=1,M;X;D 3
*01.24 T "
                                    "
*01.25 F I=1,M;X;D 4
*01.26 T "
                                                   "
*01.36 A ! "SCALE FACTOR Y",Y,"  X",X
*01.40 F I=1,M;S BU(I)=FITR(BU(I)*Y+.5);S B(I)=FITR(B(I)*X+.5)
*01.42 S I=1;T !"'",#;D 6.05;D 6.06
*01.43 F I=2,M;D 6
*01.45 I (BU(M)-1)1.50;F J=1,BU(M);T !"'"
*01.50 T #;F JJ=1,60;T "-"
*01.64 Q
*
*02.01 A !A(I);F J=1,N;A P;S SP=SP+P
*02.02 F J=1,N;A O;S SO=SO+O
*02.05 S B(I)=SP/N;S U(I)=SO/N
*
*03.01 S T=(HC+2*D/SA)*VI+A(I)*VI
*03.02 S U=T*U(I)/(B(I)+U(I));S B(I)=T*B(I)/(B(I)+U(I))
*03.03 S B(I)=B(I)-U;S B(I)=B(I)/VI;S BU(I)=B(I)/U
*03.04 T !,% 8.03,U,B(I);X
*
*04.02 T !BU(I),B(I);X
*
*06.01 I (K-BU(I)-1)6.02;F J=1,K-BU(I);T !"'"
*06.02 T #
*06.05 F JJ=1,B(I);T " "
*06.06 T "X";S K=BU(I)
**
```

PROGRAM FOR CALCULATION OF
FLAME ATOMIC ABSORPTION STANDARD CURVE
AND CORRECTION FOR DILUTION OF SAMPLES

```
C-FOCAL,69CE

01.05 E
01.07 ASK !"   N",N
01.10 FOR I=1,N;ASK !"UG/ML",X(I);SET C=C+X(I);SET D=D+X(I)↑2
01.15 F I=1,N;A !"                 % ABS",Y(I);S E=E+Y(I);D 3.15
01.20 SET A=(G-C*E/N)/(D-C↑2/N)
01.22 SET B=(E-A*C)/N
01.25 TYPE !!"  %ABS  "%8.04,A," UG/ML  +("%8.04,B,")"!!

02.00 T "FOLLOW COLON WITH TWO LETTER ID CODE"
02.10 A !ID,"% ABS",Y;S Z=(Y-B)/A
02.20 T "  UG/ML"%7.04,Z;A "  VOL BLOOD",V;T "  CORR CONC";
02.30 T Z*2*(V/(V-0.5));GOTO 2.10

03.15 S G=G+Y(I)*Y(I);S H=H+Y(I)↑2

04.15 S J=D-C↑2/N;  S K=H-E↑2/N
04.20 S VY=(K-A↑2*J)/(N-2)
04.25 S VB=VY/J;  T !"SD OF SLOPE", FSQT(VB)
04.30 S Y=10*A+B;S VX=(VY/A↑2)*(.5+1/N+(Y-E/N)↑2/A↑2*J)
04.35 T ! "SD OF  X",FSQT(VX)
04.40 T !"WHERE X=10 UG/ML AND DUPLICATE ESTIMATES ARE MADE
*
```

PROGRAM FOR CALCULATION OF OPTIMUM IRRADIATION AND DECAY TIMES FOR NEUTRON ACTIVATION ANALYSIS

```
C-FOCAL,69CF

01.01 E
01.10 A !"NO OF NUCLIDES"N,!"MIN TB"TB," INCREMENT"DB,"HOURS"
01.20 A !"MIN TD"TD," INCREMENT"DD,"HOURS";S N=N-1
01.30 F I=0,N;A !,S(I),K(I),X
01.40 S TB=TB-DB
01.50 S TB=TB+DB

05.10 S TT=TD-DD
05.20 S TT=TT+DD;S SC=0
05.30 F I=1,N;D 7.1;S SC=SC+V
05.40 S I=0;D 7.1;S RC=V/FSC1(SC)
05.50 I (RC-RD)5.6;S RD=RC;G 5.2
05.60 S TT=TT-DD;S SC=0;D 5.3;D 5.4;S RB=RC;S RD=0

06.10 I (RB-RM)6.2;S RM=RB;G 1.5
06.20 S TB=TB-DB;D 5
06.30 T !!"MAX CONDITIONS"!"TB"%4,TB,!"TD",TT;
06.40 T !"MAX RATIO"%,RB,!"AU CPM"%,V;C

07.10 S V=K(I)*(1-FEXP(-.693147*TB/S(I)))*60*FEXP(-.693147*TT/S(I))
*
```

```
 GO

NO OF NUCLIDES:17
MIN TB:290  INCREMENT:2 HOURS
MIN TD:150  INCREMENT:2 HOURS
:
                    CPM FROM
  HALF LIFE         24 HOUR
    HOURS         IRRADIATION  ISOTOPE

 0.648000E+02 :0.207501E+03::0198AU
:0.265000E+02 :0.174222E+06::0076AS
:0.359000E+02 :0.203393E+07::0082BR
:0.540000E+02 :0.572797E+02::0115CD
:0.315000E+01 :0.750000E+00::134MCS
:0.461650E+05 :0.926140E+03::0060CO
:0.128000E+02 :0.200302E+06::0064CU
:0.108200E+04 :0.112646E+06::59IRON
:0.588000E+04 :0.530656E+05::0065ZN
:0.138000E+02 :0.183777E+05::069MZN
:0.258000E+01 :0.466667E+05::0056MN
:0.670000E+02 :0.234425E+03::0099MO
:0.256000E+01 :0.183333E+02::0065NI
:0.124000E+02 :0.216932E+07::0042K
:0.448800E+03 :0.824612E+04::0086RB
:0.607200E+04 :0.539126E+06::110MAG
:0.150000E+02 :0.200785E+09::0024NA

MAX CONDITIONS
TB=  294
TD=  186
MAX RATIO= 0.576537E+00
AU CPM= 0.162923E+04*
```

APPENDIX II

DATA IN CODED FORM

Explanation of coding:
Reading from left to right the data appears in the following order.

1. Patient description: code number, sex, age, diagnosis, weight in kg, height in cm, complicatiang illness.

    Diagnosis code:
    - RA rheumatoid arthritis
    - PA psoriatic arthritis
    - OA osteoarthritis
    - D dermatomyositis
    - If blank no disease.

    Complicating illness code:
    - E endocrine disease
    - O non-endocrine disease

2. Sampling data: week of study, plasma cortisol level mcg%, plasma gold level mcg/ml. If there is more than one sample for a given patient the first line gives the average plasma cortisol level for all samples for that patient. Subsequent lines give data for individual weeks. nd= no detectable plasma gold level.

3. Gold dose information: gold dose rate mg/wk, total gold dose mg, dosage schedule mg per weeks elapsed since dose given. All doses are expressed in mg of elemental gold. Dose represents gold given prior to sample collection.

4. Side effects to gold:
    - 1=skin reaction
    - 2=mucosal reaction
    - 3=hematological reaction
    - 4=proteinuria
    - 5=chrysiasis
    - b=reaction occurred prior to study period
    - a=reaction occurred after study period
    - d=reaction occurred during study period.

Actual time when the reaction occurred is given with data.

5. Drug therapy G=gold, S=salicylate, A=indomethacin, B=propoxyphene, C=codeine, D=prednisone, E=intra-articular steroids, F=phenobarbital, H=estrogen, I=amethopterin, J=chlorpheniramine, K=glutethimide, L=chlordiazepoxide, M=diazepam, N=amitriptyline, O=caffeine, P=primidone, Q=ferrous sulfate, R=laxatives, T=orphenadrine, U=ACTH, V=vitamin-mineral supplements, W=thyroid, X=propantheline bromide, Y=betamethasone, Z=tetracycline,#=estrogen/progesterone oral contraceptives, *=penicillamine.

Drugs appearing on the first line of the patient's data were given throughout the study period. Drugs on subsequent lines were prescribed at the time indicated for that line of data.

1		2			3			4	5
Group I Gold									
2M63RA58	165		28.6					1a	GSBK
		1	39.4	4.32	25.00	342.5	25.0/1		
		2	23.0	3.94	25.00	367.5	25.0/1		
		3	32.7	4.54	25.00	392.5	25.0/1		
		4	30.3	5.80	25.00	417.5	25.0/1		
		5	37.9	6.22	25.00	442.5	25.0/1		
		6	29.6	6.03	25.00	467.5	25.0/1		
3F52RA	EO		17.7					4b	GS
		1	20.2	nd	12.50	645.0	25.0/2		
		5							PF
		9	12.0	nd	6.25	695.0	25.0/4		PFBOR
		14	23.0	0.36	5.00	720.0	25.0/5		PFBOR
		18	13.6	nd	6.25	745.0	25.0/4		PFBOR
		22	23.4	0.50	6.25	770.0	25.0/4		PFBOR
		83		2.02	6.25	1770.0	25.0/4		
4F66RA	O		18.3						GS
		1	15.5	nd	5.00	5.0	5.0/1		
		2	21.1	0.64	12.50	17.5	12.5/1		
5F15RA			9.2					12d	GS
		1	10.6	2.50	12.50	380.0	25.0/2		
		4	6.6	3.92	25.00	455.0	25.0/1		
		5	11.1	4.08	25.00	480.0	25.0/1		
		6	11.4	6.50	25.00	505.0	25.0/1	12	
		7	6.7	3.66	12.50	505.0	25.0/2		
		10	11.7	0.98	5.00	505.0	25.0/5		
		17	5.0	2.54	12.50	605.0	25.0/2		
		23	14.4	1.88	6.25	655.0	25.0/4		
7F46RA63	174		20.0					2d	GS
		1	20.1	1.44	6.25	280.0	12.5/2		
		3							DN
		5	21.3	1.18	6.25	305.0	12.5/2		
		7	17.2	2.10	6.25	317.5	12.5/2		
		9	19.8	1.87	6.25	330.0	12.5/2		
		17	18.8	1.18	6.25	367.5	12.5/2		E
		19	23.3	1.01	6.25	380.0	12.5/2	2	
		20	19.6	1.30	4.17	380.0	12.5/3		

		22	22.8	1.90	6.25	392.5	12.5/2		E
9F52RA	EO		38.5					2a	G
		1	41.1	3.14	25.00	92.5	25.0/1		
		2	39.0	4.07	25.00	117.5	25.0/1		
		4	49.0	3.97	25.00	167.5	25.0/1		
11F51RA			14.0						GS
		1	5.7	1.13	12.50	42.5	25.0/1		
		4	17.8	3.66	25.00	117.5	25.0/1		
		5	11.5	4.65	25.00	142.5	25.0/1		
		6	13.8	6.25	25.00	167.5	25.0/1		
		7	7.2	5.80	25.00	192.5	25.0/1		
		8	18.4	5.36	25.00	217.5	25.0/1		
		9	10.3	5.17	25.00	242.5	25.0/1		
		10	31.7	5.88	25.00	267.5	25.0/1		
		14	16.6	5.31	25.00	342.5	25.0/1		
		16	9.3	3.67	12.50	367.5	25.0/2		
		17	14.4	4.68	25.00	392.5	25.0/1		
		19	10.4	5.50	25.00	442.5	25.0/1		
		20	12.8	5.84	25.00	467.5	25.0/1		
		22	19.4	7.11	25.00	517.5	25.0/1		
12F53RA			20.3					14b	GS
		1	20.3	nd	6.25	1592.5	25.0/4		
		89		2.91	17.50	1967.5	25.0/1.4		
13F34RA60	171		16.0					4b	GS
		1	23.3	0.77	4.17	597.5	12.5/3		
		4	17.3	nd	4.17	610.0	12.5/3		
		8	17.9	0.48	3.12	622.5	12.5/4		
		12	9.9	0.65	3.12	635.0	12.5/4		
		16	22.2	0.55	6.25	660.0	25.0/4		
15M42RA66	180		16.7						GS
		1	26.0	nd	5.00	5.0	5.0/1		
		3	10.6	1.18	12.50	42.5	12.5/1		
16M18PA48	166		13.9					1a	G
		1	27.7	nd	5.00	5.0	5.0/1		
		3	14.7	nd	6.25	17.5	12.5/2		
		4	11.2	1.19	12.50	30.0	12.5/1		
18F50RA			20.8						GS
		1	17.5	1.85	6.25	475.0	25.0/4		
		9	24.9	0.66	6.25	525.0	25.0/4		
		14	21.4	0.35	5.00	550.0	25.0/5		
		18	22.8	0.85	6.25	575.0	25.0/4		
19M20RA		1	15.8	3.92	25.00	167.5	25.0/1	12a	GS

20M59RA		0	15.2					14b	GS
		1	11.4	nd	6.25	1470.0	25.0/4		R
		5	11.5	nd	6.25	1495.0	25.0/4		R
		9	14.5	0.88	6.25	1520.0	25.0/4		
		12	16.6	nd	6.25	1545.0	25.0/4		
		17	23.4	1.93	6.25	1570.0	25.0/4		
21M64RA			11.3						GS
		1		0.34	6.25	592.5	25.0/4		
		5	11.4	nd	6.25	617.5	25.0/4		
		9	12.0	nd	6.25	642.5	25.0/4		
		13	11.1	0.51	6.25	667.5	25.0/4		
		66		0.70	5.00	942.5	25.0/5		
22F50RA50	154		16.7					1bd	GS
		1	6.9	0.85	6.25	382.5	12.5/2		
		5	12.2	1.18	6.25	407.5	12.5/2		
		7	26.1	2.08	6.25	420.0	12.5/2		
		9							J
		14	15.7	2.40	6.25	457.5	12.5/2	1	
		22	23.4	1.25	1.25	457.5	12.5/10		
23M39RA			17.2					3b	GS
		1	10.3	nd	6.25	735.0	25.0/4		
		6	14.1	0.78	5.00	760.0	25.0/5		
		10	17.1	nd	6.25	785.0	25.0/4		
		14	20.8	0.95	6.25	810.0	25.0/4		
		18	27.4	0.52	6.25	835.0	25.0/4		
		22	13.4	nd	6.25	860.0	25.0/4		
		105		2.39	5.00	1360.0	25.0/5		
27F55RA			13.4					1b5	GS
		1	8.4	nd	12.50	865.0	12.5/1		
		20	23.0	0.49	3.12	940.0	12.5/4		
28F39RA		0	17.4						G
		1	15.0	1.67	12.50	400.0	25.0/2		
		5	18.8	2.75	12.50	475.0	25.0/2		
		7	19.3	2.09	12.50	500.0	25.0/2		
		9	22.6	1.87	12.50	525.0	25.0/2		
		14	17.9	2.17	12.50	575.0	25.0/2		
		16	10.1	3.87	25.00	625.0	25.0/1		
		18	18.6	2.48	12.50	650.0	25.0/2		
29F30RA66	165		29.0						GS
		1	25.7	2.23	5.00	425.0	25.0/5		
		5	36.1	1.50	12.50	500.0	25.0/2		
		7	26.6	2.59	12.50	525.0	25.0/2		
		9	36.0	1.88	12.50	550.0	25.0/2		
		12							MN
		14	28.0	1.37	12.50	600.0	25.0/2		

			16	31.4	1.87	12.50	625.0	25.0/2		
			18	34.1	1.50	12.50	650.0	25.0/2		
			20	32.0	2.61	12.50	675.0	25.0/2		
			22	24.3	1.90	12.50	700.0	25.0/2		B
30F61RA				24.2	nd	1.47	512.5	25.0/17	1b	GS
31M52RA		0		10.3					1d	GS
			1	12.7	0.78	6.25	587.5	25.0/4	1	
			5	6.6	0.98	6.25	612.5	25.0/4		
			9	13.7	nd	6.25	637.5	25.0/4		
			12	15.0	0.77	6.25	662.5	25.0/4		
			17	8.7	1.19	6.25	687.5	25.0/4		
			101		0.67	5.00	962.5	25.0/5		
32F50RA				32.9	0.61	25.00	700.0	25.0/1		GS
33F38RA				18.7					5	GS
			1	17.9	0.58	6.25	5275.0	25.0/4		
			13	20.1	1.09	6.25	5350.0	25.0/4		
			17	26.2	0.37	6.25	5375.0	25.0/4		
			21	25.2	nd	6.25	5400.0	25.0/4		
			69		0.92	5.00	5675.0	25.0/5		
34F64RA				25.0						GS
			1	29.4	1.45	25.00	500.0	25.0/1		
			3	22.6	2.13	12.50	525.0	25.0/2		
			5	26.3	2.12	12.50	550.0	25.0/2		
			7	16.6	2.01	12.50	575.0	25.0/2		
			16	27.6	1.01	6.25	650.0	25.0/4		
			19	33.6	2.24	8.33	675.0	25.0/3		
36F45RA				17.7					1b	GS
			1	12.4	nd	6.25	252.5	12.5/2		
			4	19.5	0.61	4.17	265.0	12.5/3		
			7	25.1	1.04	4.17	277.5	12.5/3		
			10	19.4	0.46	4.17	290.0	12.5/3		
			13	8.8	nd	4.17	302.5	12.5/3		
			17	26.1	nd	3.12	315.0	12.5/4		
			21	14.4	0.54	3.12	327.5	12.5/4		
37F55RA		0		15.6						GS
			1	14.1	0.71	6.25	1225.0	25.0/4		
			8	21.6	0.64	3.57	1250.0	25.0/7		
			12	14.9	1.94	6.25	1275.0	25.0/4		
38F65RA48	152	0		14.5					4b1d	GS
			1	10.8	nd	0.35	615.0	25.0/71		
			5	27.9	nd	0.33	616.0	25.0/72		
			6						1	

			9	8.4	nd	0.32	615.0	25.0/76		E
			13	9.7	nd	5.00	620.0	5.0/1		
			14	30.5	0.57	12.50	632.5	12.5/1		
			15							E
			16	14.2	1.07	6.25	645.0	12.5/2		
			17	7.4	1.75	12.50	657.5	12.5/1		
			18	14.2	1.98	12.50	670.0	12.5/1		
			20	13.4	2.61	12.50	695.0	12.5/1		
			22	9.9	2.24	6.25	707.5	12.5/2		
39M63RA68	174	O		17.0					1b	GS
			1	10.0	nd	6.25	1130.0	25.0/4		
			5	17.7	0.86	6.25	1155.0	25.0/4		
			9	23.1	0.82	6.25	1180.0	25.0/4		
			13	10.0	0.68	6.25	1205.0	25.0/4		
			17	31.1	1.18	6.25	1230.0	25.0/4		
			21	15.8	0.89	6.25	1255.0	25.0/4		
40F43RA				21.4	nd	5.00	5.0	5.0/1		GS
41F63RA41	152	O		10.8					14b	GS
			1	9.0	nd	5.00	4550.0	25.0/5		
			6	5.7	1.31	6.25	4600.0	25.0/4		
			10	11.2	0.97	6.25	4625.0	25.0/4		
			16	20.6	0.67	4.17	4650.0	25.0/6		
42F50RA				26.2						GS
			1	16.1	0.55	12.50	707.5	25.0/2		
			9							C
			13	38.9	0.37	6.25	782.5	25.0/4		
			17	24.9	0.55	6.25	807.5	25.0/4		
43F48RA				10.6					1b2a	GS
			1	6.1	nd	6.25	855.0	25.0/4		
			6	12.8	0.77	5.00	880.0	25.0/5		
			14	19.4	nd	6.25	930.0	25.0/4		
			18	8.7	0.52	6.25	955.0	25.0/4		
44M59RA		O		17.0					12b	GS
			1	10.8	0.75	5.00	5.0	5.0/1		
			3	23.8	1.94	25.00	42.5	25.0/1		
45F46RA				29.4	nd	5.00	5.0	5.0/1	1a	G
46F44RA		O		18.3					1b	GS
			1	12.7	2.34	25.00	290.0	25.0/1		
			4	12.3	2.28	12.50	340.0	25.0/2		D
			5	18.8	3.72	25.00	365.0	25.0/1		
			6	14.7	4.56	25.00	390.0	25.0/1		
			7	24.0	5.80	25.00	415.0	25.0/1		
			8	18.7	4.60	25.00	440.0	25.0/1		

		9	20.2	4.54	25.00	465.0	25.0/1		
		10	22.7	5.73	25.00	490.0	25.0/1		Q
		18	24.7	1.75	8.33	565.0	25.0/3		
		21	17.5	1.62	8.33	590.0	25.0/3		
47F58RA			17.2					24d	GS
		1	5.6	1.27	25.00	67.5	25.0/1		
		4	19.6	3.36	25.00	142.5	25.0/1		
		6	18.8	5.05	25.00	192.5	25.0/1		
		7	10.0		25.00	217.5	25.0/1		
		9	24.6	3.78	25.00	267.5	25.0/1		
		12						24	
		13	8.8	2.32	8.33	317.5	25.0/3		
		14	34.0	4.17	25.00	342.5	25.0/1		
		16	16.5	2.26	12.50	367.5	25.0/2	24	
		17	13.6	2.80	25.00	392.5	25.0/1		
		18	12.6	3.49	25.00	417.5	25.0/1		
		20	13.8	2.35	12.50	442.5	25.0/2	24	
		21	23.2	3.32	12.50	455.0	12.5/1		
		22	21.7	1.91	12.50	467.5	12.5/1	24	
		23	14.6	1.54	6.25	467.5	12.5/2		
48F 6RA			8.4					1d	GS
		1	7.0	0.72	5.00	27.2	5.0/1		
		4						1	
		23	7.2	1.19	2.50	117.5	5.0/2		
49F44RA55	163EO		16.0					1d	GS
		1	20.8	0.46	2.78	75.0	25.0/9		
		5	16.5	1.75	6.25	100.0	25.0/4		
		9	10.6	1.19	6.25	125.0	25.0/4	1	H
50M46RA			33.6	nd	6.25	1125.0	25.0/4	14b	GS
51F72RA60	156 O		30.2					1b5	GS
		1	20.8	nd	6.25	1192.5	25.0/4		
		5	46.3	0.54	6.25	2217.5	25.0/4		
		9	31.5	0.46	6.25	2242.5	25.0/4		
		13	25.4	0.47	6.25	2267.5	25.0/4		
		17	35.0	0.64	6.25	2292.5	25.0/4		
		21	26.6	0.55	6.25	2317.5	25.0/4		
		69		0.83	2.27	2492.5	25.0/11		
52M38RA86	178 O		17.9					2a	GS
		1	18.7	nd	5.00	5.0	5.0/1		
		2	10.4	0.70	12.50	17.5	12.5/1		
		3	13.5	1.80	25.00	42.5	25.0/1		
		4	11.8	2.34	25.00	67.5	25.0/1		
		5	10.1	2.62	25.00	92.5	25.0/1		
		6	7.5	3.10	25.00	117.5	25.0/1		
		7	53.8	3.30	25.00	142.5	25.0/1		

53F55RA	0		13.7					4d	GSR
		1	3.7	nd	6.25	17.5	12.5/2		
		3						4	
		5	15.2	nd	2.08	17.5	12.5/6		
		6	14.0	1.57	12.50	30.0	12.5/1		
		8	15.4	0.26	4.13	30.0	12.5/3		
		9	9.4	1.48	12.50	42.5	12.5/1		
		10	27.7	1.21	6.25	42.5	12.5/2		
54F56RA			9.4						GS
		1	11.5	3.69	25.00	417.5	25.0/1		
		3	14.7	5.14	25.00	467.5	25.0/1		
		4	16.4	6.26	25.00	492.5	25.0/1		
		6	7.0	2.75	12.50	517.5	25.0/2		
		7	3.9	4.25	25.00	542.5	25.0/1		
55F61RA			16.5					2b	GS
		1	13.5	1.04	6.25	842.5	25.0/4		
		5	21.5	0.74	6.25	867.5	25.0/4		
		9	27.3	nd	6.25	892.5	25.0/4		
		13	6.8	1.46	6.25	917.5	25.0/4		
		17	13.2	1.21	6.25	942.5	25.0/4		
56F31RA	0		13.9						G
	-	1	21.8	nd	5.00	5.0	5.0/1		
		2	17.9	1.88	12.50	17.5	12.5/1		
		3	13.8	1.21	12.50	30.0	12.5/1		
8F55RA64	162E		25.8						
		1	25.6	0	0	0	0		
		2	31.2	0.75	5.00	5.0	5.0/1		G
		3	29.3	0.54	12.50	17.5	12.5/1		G
		4	35.8	2.63	25.00	42.5	25.0/1		G
		5	22.6	2.95	25.00	67.5	25.0/1		G
25F42RA			28.5						S
		1	19.2	0	0	0	0		
		2	25.3	nd	5.00	5.0	5.0/1		G
		5	22.1	1.36	25.00	42.5	25.0/1		G
		6	23.2	2.69	25.00	67.5	25.0/1		G
		8	31.1	2.06	12.50	92.5	25.0/2		G
		9	41.1	1.98	25.00	117.5	25.0/1		G
		10	40.1	2.97	25.00	142.5	25.0/1		G
		13	31.6	3.69	25.00	217.5	25.0/1		
		14	23.7	3.30	25.00	242.5	25.0/1		G
26M37PA			27.7					1d	S
		1	39.4	0	0	0	0		
		2	35.5	nd	5.00	5.0	5.0/1		G
		3						1	G
		4	31.2	nd	6.25	17.5	12.5/2		G

		5	22.7	2.35	25.00	42.5	25.0/1		G
		7	18.2	1.91	12.50	67.5	25.0/2		G
35F52RA	EO		18.7						S
		1	31.2	0	0	0	0		E
		2	14.4	0.46	5.00	5.0	5.0/1		G
		6	25.4	3.38	25.00	67.5	25.0/1		G
		8	24.4	4.12	25.00	117.5	25.0/1		G
		10	19.8	4.44	25.00	167.5	25.0/1		G
		11	19.8	3.91	25.00	192.5	25.0/1		G
		12	13.2	3.97	25.00	217.5	25.0/1		G
		13	7.5	6.09	25.00	242.5	25.0/1		G
		14	19.8	5.39	25.00	267.5	25.0/1		G
		15	24.5	5.35	25.00	292.5	25.0/1		G
W F61RA				0.60		>2500.0		1b5	G
J F61RA52 161						17.5			GS
N F RA	E		18.6			17.5			G

Group II No Gold

1M44PA			18.6		I
		1	19.5	0	
		2	15.2	0	
		3	26.3	0	
		4	22.0	0	
6F39PA			9.2		I
		1	14.3	0	
		5	5.8	0	
10F66OA		1	15.9	0	A
14F44RA	EO	1	26.5	0	SBLT
17M16RA		1	12.2	0	S
24F17D	EO		23.2		SDI
		1	25.0	0	
		2	15.9	0	
		11	28.6	0	
57M50PA		1	17.6		UV
58M13RA		1	3.7		S
59F48RA		1	2.2		SV

60M51RA	O	1	20.2	S
61F52RA		1	1.6	S
62F65RA	EO	1	16.0	SHVWX
63M65RA		1	8.6	SA
64M53RA		1	14.6	S
65F55RA		1	7.7	S
66F27RA		1	9.4	SEY
67M72RA	O	1	12.8	SDE
68M11RA		1	2.8	S
69F64RA		1	13.7	A
70F46RA	O	1	23.2	S

Group III Control

71M28		1	3.7	
72F19		1	11.6	Z
73F19		1	6.0	
74F22		1	18.0	
75F20	E	1	15.7	
76F24		1	15.7	#
77F19		1	10.0	#
78F20		1	10.2	Z
79M27		1	2.4	
80F27		1	18.3	
81M39		1	2.4	
82M35		1	6.5	
83M25		1	8.7	

84F22	1	22.4
85F25	1	15.4
86M31	1	9.2
87M24	1	22.2
88M28	1	14.1
89F23	1	11.0
90M29	1	20.6
91F25	1	9.2